# Using Genomic Transgenes and the CRISPR/Cas9 Gene Editing System to Understand How Hedgehog Signaling Regulates Costal2 and Cubitus Interruptus in *Drosophila melanogaster*

Jamie Little

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ABSTRACT

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The Hedgehog protein (Hh) is a morphogen that is necessary for cell survival, growth and patterning in flies and mammals. In germline cells, alterations in the Hh signaling pathway can result in developmental disorders; in somatic cells, misregulation of the Hh signaling pathway can result in cancer. Most components of the signaling pathway were identified by genetic screens in Drosophila that were later found to be conserved in mammals. In the presence of the Hh signal, multiple Hh signaling components interact to mediate the induction of Hh target genes. In flies, Cubitus Interruptus (Ci) is the singular transcription factor of the pathway that is regulated by multiple upstream components of the pathway including Costal2 (Cos2). Cos2 is a scaffold protein that can both positively and negatively regulate Hh signaling by binding to Ci and various kinases such as Fused (Fu).

We disrupted the binding of Cos2 to Fu using a physiological expressed genomic Costal2 transgene (gCosΔFu) and found that Fu must bind to Cos2 to promote efficient processing and activation of full-length Ci (Ci-155). Fu was thought to activate Ci-155 by phosphorylating Cos2 at sites S931 and S572, but we found that gCosS931A and gCosS572A did not reduce Ci activity in the fly wing disc. Instead, we hypothesize that Fu could directly phosphorylate Ci-155 or another unknown protein. To investigate if another protein was involved we developed a Hh sensitized genetic screen.

We obtained multiple “hits” from the genetic screen but we did not find an obvious candidate that could be a substrate for Fu. Instead, we identified Mago Nashi and Srp54 which we found to be involved with the post-transcriptional regulation of ci RNA. We confirmed the existence of ci isoforms A and B and found that knockdown of Mago Nashi, resulted in an altered splicing pattern while knockdown of Srp54 reduced ci RNA levels. Mago Nashi inhibition and intronless Ci reduced Ci-155 protein levels, which suggests efficient splicing is necessary for normal Ci-155 levels. Furthermore, we found that reduced Ci-155 levels only affected Ci activity in sub-optimal Hh signaling conditions.

In order to further dissect the mechanism Ci processing, activation and stabilization, we used physiologically expressed genomic Ci (gCi) and CRISPR Ci variants (crCi). First we examined Ci-S849A, which prevents Ci processing and we found that in the absence of processing, Ci-155 levels are uniformly high throughout the wing disc. Cos2 and PKA are necessary for Ci processing but we wanted to know if they had an additional role in Ci silencing We found that Cos2 but not PKA can silence and stabilize Ci-155 in the absence of processing. Activated Fu in the Ci-S849A wing disc highly activated and destabilized Ci-155, which was similar to Hh signaling at the AP Border.

To test if Ci is the direct target of Fu, we are testing physiologically expressed Ci with point mutations and deletions that are near the Suppressor of Fused (Su(fu)) binding site to examine whether they are unresponsive to activated Fu. Su(fu) binds to Ci-155 to stabilize Ci-155 levels and inhibit Ci activity, but the mechanism is not well understood. We developed Ci transgenes that have altered Su(fu) binding to determine if Su(fu) inhibits Ci by cytoplasmic anchoring, co-repressor recruitment, or by blocking a co-activator.

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Glossary

Clones (wing disc): A genetic technique used to create a subset of cells in the wing disc that have a specific genetic alteration different from the rest of the disc. These cells can be induced by either positive or negative marking using the Flp/FRT recombinase system. In positive marking, cells that co-express GFP will contain the mutation of interest. In negative marking, the entire disc will express GFP whereas the absence of GFP marks the alteration.

Notable Hh signaling component clones:

$ci^{94}$ + p[Ci] clones: An extra copy Ci is expressed throughout fly and null Ci ($ci^{94}$) clones are induced

*cos2* clones: Loss of Costal2 blocks processing and induces moderate *ptc-lacZ* in the Anterior, reduces ptc-lacZ at the AP border , and highly elevates Ci-155 levels in the Anterior and AP Border

Gap-Fu clones (Membrane Bound Active Fused): Activated Fused activates Ci-155 and induces moderately high *ptc-lacZ* in the Anterior as well as slightly elevates Ci-155 levels

*pka* clones: Loss of Protein Kinase A blocks processing and induces moderate *ptc-lacZ* as well as highly elevates Ci-155 levels in the Anterior

*ptc* clones: Loss of Patched allows for activation of Smo and induces high *ptc-lacZ* in the Anterior and AP Border as well as moderately high Ci-155 levels

*slimb* clones: Loss of Slimb blocks processing, elevating Ci-155 levels and moderately increasing *ptc-lacZ*

*smo* clones: Loss of Smoothened prevents Hh response and decreases *ptc-lacZ* and Ci-155 at the AP Border

*Su(fu)LP + cos/pka* clones: Loss of Costal2/PKA in null Su(fu) fly induces higher *ptc-lacZ* in the Anterior than *cos* or *pka* alone.

Costal2 (Cos2): A scaffold protein that positively and negatively regulates Hh signaling. Costal2 promotes Ci-155 processing by binding to kinases CK1, GSK3, and PKA which can then phosphorylate full-length Ci-155. Slimb, the E3 ubiquitin ligase recognition

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| | component, binds to phosphorylated Ci-155 promoting partial proteolysis to Ci-75. Costal2 positively regulates Hh by binding to Fused and promoting Ci activation. |
| CRISPR-Cas9: | A gene editing system that allows for direct modification of a gene of interest. |
| Cubitus Interruptus (ci): | The singular transcriptional factor in the Hedgehog Signaling pathway in Drosophila. Its full-length form (Ci-155) can be 1) Activated 2) Processed into a repressor (Ci-75) form 3) Completely Proteolyzed or 4) Post-transcriptionally regulated<br><br>Notable Ci mutants:<br>*Ci-S849A*: Serine 849 is mutated to an alanine which prevents phosphorylation by Ck1. This prevents Slimb from binding and blocks processing. There is elevated Ci-155 levels and minimal elevation of *ptc-lacZ* in the anterior<br><br>*Ci-SYAAD:* Mutating SYGHI to SYAAD in exon 3 has been shown to block Sufu binding *in vitro*. This transgene has been shown to partially block Sufu binding by moderately rescuing ptc-lacZ in a fused mutant. There is also decreased Ci-155 levels comparable to loss of Sufu alone.<br><br>*CiΔ230-272* and *CiΔ175-346*: Deletion regions that contains SYGHI. Loss of these amino acids result in expanded anterior and increased lethality in the fly. They show normal *ptc-lacZ* and reduced Ci-155 levels.<br><br>*CiΔCORD*: Deletion of the CORD region (aa 939-1065) showed no lethality defects in the flies. The CORD, CDN and Zn Finger regions on Ci bind to Costal2. |
| Fused (Fu): | A protein kinase that is necessary for Ci Activation. Loss of Fu in the wing disc reduces *ptc-lacZ* and elevates Ci-155 levels. Fu also contributes to efficient Ci-155 processing. |
| Genomic Transgene: | The entirety of a gene cloned into an ATT vector. It includes the upstream and downstream regulatory elements as well as the exons and introns. |
| Hedgehog (Hh): | A morphogenic signaling protein that binds to the receptor Patched to promote a downstream Hh signaling response in flies and mammals. |

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| Ihog and Boi: | Patched co-receptors in Drosophila |
| Patched (Ptc): | Hedgehog signaling protein receptor. Patched inhibits the activity of another transmembrane protein, Smoothened. When Hh binds to Patched, Patched activity is inhibited and Smoothened is activated. Patched is also a target gene that is expressed when Hh signaling is active. |
| PKA/Gsk3/Ck1: | Kinases that are responsible for phosphorylating Slimb and promoting Ci-155 processing to Ci-75. |
| *ptc-lacZ:* | A commonly used reporter to monitor Hh Signaling Activity. The patched promoter is fused to *lacZ*. |
| Roadkill/HIB (Rdx): | A BTB recognition component of the Cul-3 Ubiquitin Ligase Complex that promotes the complete degradation of Ci-155 at high levels of Hh signaling. |
| Slimb: | Is the F-Box recognition component of the SCF (Skp1, Cul-1, F-Box containing) Complex in Drosophila that recognizes phosphorylated Ci-155 |
| Smoothened (Smo): | Transmembrane protein that promotes Ci activation and induction of Hh target genes in response to the Hh signal. |
| Suppressor of Fused (Su(fu)): | Inhibits Ci activity and stabilizes Ci-155 levels. Loss of Su(fu) reduces Ci-155 levels but has no effect on *ptc-lacZ*. Loss of Su(fu) rescues the loss of Fused. |
| UAS/Gal4 System: | A tool used in Drosophila and engineered from yeast that induces the expression of a gene in a specific type of tissue. The Upstream Activating Sequencing (UAS) is fused to the cDNA of a gene of interest such as UAS-GFP. A tissue specific promoter is fused to the Gal4 yeast activating protein such as C765(a wing specific promoter)-Gal4. So that when UAS-GFP flies are crossed to C765-Gal4 flies, their progeny will express GFP exclusively in the wing disc. |
| Wing Disc: | The wing disc is divided into different compartments, the Anterior which receives the Hh signal and promotes the downstream Hh response and the Posterior which is responsible for producing the Hh signal. The boundary between the two compartments is the Anterior/ Posterior Boundary (AP Border) |

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# Chapter 1

# Introduction

Hedgehog (Hh) signaling proteins guide development and help maintain adult tissue homeostasis in both invertebrates and vertebrates (Ingham and McMahon 2001). Aberrant Hh protein production, distribution, and responses are common causes of developmental birth defects and cancer (Low and de Sauvage 2010). We study Hh signaling in order to identify its various components and how they function to regulate the levels of pathway activation, but there are still many aspects that are not well understood. The discovery of these unknowns could offer profound insight into mechanisms of certain Hh related diseases.

In 1980, Nusslein-Volhard and Wieschaus used the segmented Drosophila larvae to perform a genetic screen in search of mutants that would alter the segment pattern and their corresponding bristles also known as denticles. They found several mutants with altered segment patterning: 1) segment polarity mutants which had the normal number of segments but part of each segment was deleted and the remaining segment was a mirror image duplication 2) pair rule mutants which had a deletion in every other segment and 3) gap mutants which had multiple deletions of adjacent segments. *hedgehog* was identified from one of the segment polarity mutants; the *hh* mutant was shorter than wild type and had a great number denticles causing it to look "hairy" and resemble the animal hedgehog from which it got its name (Nusslein-Volhard and Wieschaus 1980). Further research showed this gene to code for a signaling ligand that is an essential regulator of multiple tissues in the fly such as the eye, wing, and leg.

While there is only one singular gene for Hh in Drosophila, there are three paralogs in mammals known as *sonic hedgehog* (*shh*), *desert hedgehog* (*dhh*), and *indian hedgehog* (*ihh*). In 1993, these Hh genes were isolated from mice, chickens and zebrafish (in zebrafish there are at least five different *hh* genes); they were found to act as morphogens and critical regulators of tissue development (Krauss, Concordet et al. 1993) (Echelard, Epstein et al. 1993) (Riddle,

Johnson et al. 1993). Shh is the best studied of the three ligands due to its higher prevalence and its role in determining cell fates in the central nervous system (Pathi, Pagan-Westphal et al. 2001); it can stimulate cell division in the adult mouse forebrain and maintain the population of quiescent neural stem cells allowing for self-renewal and differentiation (Ahn and Joyner 2005).

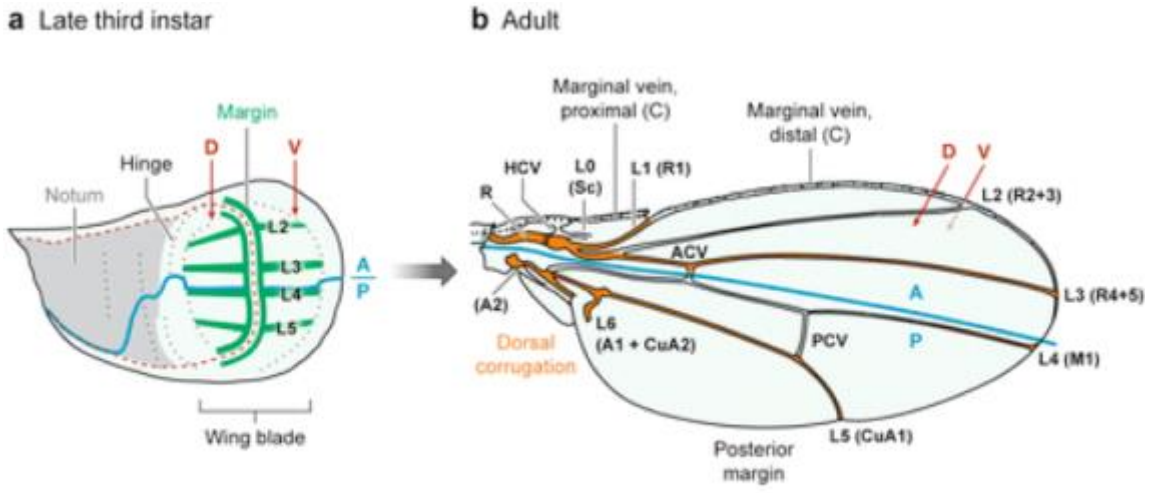
Misregulation of Hh signaling is the cause of many cancers including basal cell carcinoma and medulloblastoma (Lou, Schomaker et al. 2016). Basal cell carcinoma is the most common form of skin cancer and it is caused by excessive Hh pathway activation (Yin and Esmaeli 2017); it can be artificially induced by ectopic expression of Shh (Oro, Higgins et al. 1997). Medulloblastoma is a malignant brain cancer formed in the posterior fossa mainly in young children (Packer, Goldwein et al. 1999). Currently medulloblastoma and basal cell carcinoma can be treated with Hh specific drugs (vismodegib, Hh Antag, LDE225, IPI-926, and PF-5276857) that target specific components of the pathway (Rimkus, Carpenter et al. 2016).

Hh misregulation is also a leading cause for developmental abnormalities. In 1968, scientists found a group of one-eyed sheep that had become cyclops-like because they were eating flowers containing a poison (cyclopamine) that naturally targets Smoothened, a positive regulator of Hh signaling (Herper 2005). When cyclopamine inhibited Hh signaling in the developing sheep, a singular eye developed because Shh is responsible for forming midline structures. In human disease, mutations in *Shh* result in holoprocencephaly, which is the general defect that includes the most severe form of cyclopia (Dubourg, Bendavid et al. 2007, Xavier, Seppala et al. 2016). Developmental abnormalities in Hh signaling can also include polydactylism, a physical anomaly that adds an extra digit to a human's hand/foot (Matsubara, Nakano et al. 2016).

Understanding the basic molecular mechanisms of Hh communication is the first step in combating these various Hh related disorders. Many conserved Hh components were initially found in *Drosophila melanogaster* and then found to have a mammalian ortholog. Hence, it is important to understand the precise molecular mechanisms and proteins involved in the pathway in Drosophila.

**Studying Hh Signaling in the Drosophila Wing**

Hh signaling is critical for the appropriate development of various systems in the fly. We study the regulation of the Hh pathway in wing development by examining both the imaginal larval wing disc and the adult wing (Strigini and Cohen 1997). In the adult wing, the intervein spacing between veins L3 and L4 reveals deficits in Hh signaling, when the pathway is less active these veins are closer together (Figure 1B). For more detailed analysis, we study the larval imaginal wing disc because we can immunostain for Hh signal transduction components and target gene activity/induction (Figure 1A).




Blair SS. 2007.
Annu. Rev. Cell Dev. Biol. 23:293–319

**Figure 1 Compartmental Boundaries of the Larval Imaginal wing disc Adult Wing:** adapted from (Blair 2007) a) Diagram of the larval wing disc divided into multiple compartments: Dorsal compartment of the wing pouch is represented by D (red) on the left next to the Dorsal/Ventral boundary and the Ventral compartment on the right represented by a V (red). L2-L5 represent pre-wing veins that develop into the corresponding adult wings. The anterior compartment is on top represented by A (blue), below is the posterior compartment marked by P (blue) and the dividing region is the anterior/posterior border (AP Border) b) The imaginal wing disc matures into the adult wing. We are focusing on the intervein spacing between veins L3 and L4 to study Hh signaling.

In the first instar larvae, the wing disc is divided into different compartments with precisely defined regions. The dorsal/ventral boundary is determined by the expression of the apterous gene which is expressed in the dorsal compartment (Blair 1993, Diaz-Benjumea and Cohen 1993, Williams, Paddock et al. 1993). The anterior/posterior boundary (AP Border) is determined by the patterned expression of the homeobox transcription factor Engrailed in the posterior compartment (Kornberg, Siden et al. 1985). Engrailed stimulates the expression of *hh* while inhibiting the expression of *cubitus interruptus (ci)* and *patched (ptc)*; as a consequence, *engrailed* and *hh* are expressed in posterior compartment cells, *ci* and *ptc* are expressed in anterior cells, and *ptc* is upregulated by Hh at the AP Border. (Tabata, Eaton et al. 1992) (Sanicola, Sekelsky et al. 1995). The Hh ligand is produced in the posterior and migrates to the anterior region to control the gene expression of Hh target genes at the AP Border (Basler and Struhl 1994).

Hh signaling activity can be measured using immunostaining of Hh target gene products in anterior cells close to the source of Hh ligand, the AP Border (Figure 2). Hh is a morphogen that induces gene targets including *engrailed, ptc, decapentaplegic (dpp), and collier* (Crozatier, Glise et al. 2003) (Nahmad and Stathopoulos 2010). In the adult wing, Hh activity is responsible for the patterning between veins L3-L4 by inducing the expression of the transcription factor *collier* in a concentration dependent manner (Vervoort, Crozatier et al. 1999). *dpp* is expressed at moderate to low levels of Hh signaling and is a critical morphogen necessary for patterning and growth in the imaginal discs (Affolter and Basler, 2007). *engrailed* is induced at only the highest levels of Hh signaling and *patched* is induced at moderate levels Hh signaling (Affolter and Basler 2007). *patched* expression in the wing disc is conveniently monitored by a *ptc-lacZ* reporter gene, which is expressed strictly in anterior cells; Engrailed antibody staining is always combined with *ptc-lacZ* or Ci protein staining to distinguish anterior cells from posterior cells that express *engrailed* independent of Hh signaling.

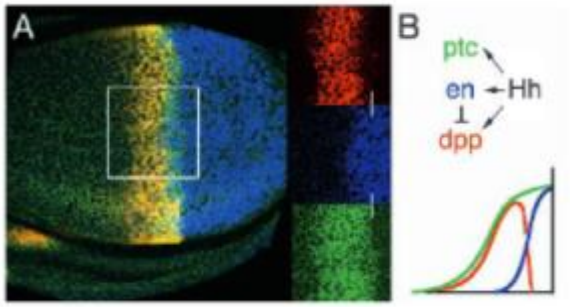



**Figure 2 Immunostaining in the Larval Wing Disc: adapted from (Strigini and Cohen 1997)** A) The image on the left is a merged image of Patched protein (green), *dpp-lacZ* expression (red) and Engrailed protein (blue) staining in the Drosophila wing disc at the AP Border. *patched* is expressed at the AP Border while *engrailed* is induced at the highest

levels of Hh signaling and is ubiquitiously throughout the posterior by a separate mechanism than Hh signaling; in order to analyze Hh-dependent expression of *engrailed*, we co-stain with Patched or *ptc-lacZ* to mark the AP Border.

*Genetic Techniques*

There are several genetic techniques that can be employed to study the Drosophila wing disc including: UAS/Gal4, genomic transgenes, CRISPR and the Flp/Frt Recombination,

*UAS/Gal4*

The UAS/Gal4 system was engineered from yeast to use in Drosophila to study the consequences of overexpressed, ectopically expressed, or knocked down proteins in a specific tissue; the Gal4 protein will only activate genes that are regulated by a region contacting multiple Gal4 binding sites, the "UAS (Upstream Activating Sequence)". This promoter sequence is fused to a gene or RNAi of interest that will be expressed in the presence of the ectopic yeast Gal4 activating protein. The *Gal4* gene is fused to a tissue specific promoter and expressed exclusively in that tissue allowing for the Gal4 "driver" protein to activate the corresponding UAS gene/RNAi target. We have used the UAS/Gal4 system to study Hh signaling using 1) C765 Gal4, a wing disc specific driver that is expressed moderately and evenly throughout the imaginal wing disc, 2) MS1096 Gal4, a highly-expressed wing disc driver in the dorsal compartment, and 3) apterous-Gal4, another highly expressed wing disc driver in the dorsal compartment. The UAS/Gal4 is sensitive to temperature so we can control the temperature to attempt to get the appropriate expression of a specific UAS-gene (Figure 3).

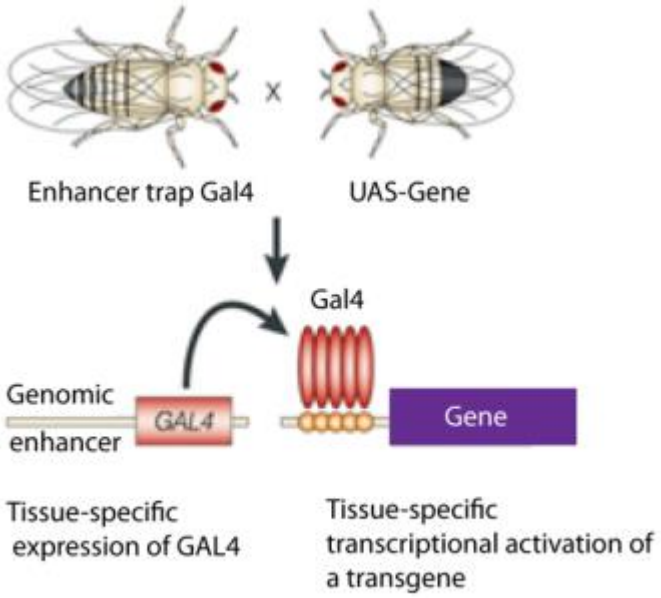



**Figure 3 UAS/Gal4:** (adapted from Muqit and Feany, 2002) In this diagram, the male fly on the right contains a UAS-transgene, the female fly on the left has a Gal4 protein driven by a tissue specific promoter. Their progeny will have both the UAS-transgene and the Gal4 in their DNA which allows for the Gal4 protein to activate the UAS- gene in a tissue of interest (Muqit and Feany 2002).

*Genomic Transgenes*

Genomic transgenes include the upstream/downstream regulatory regions as well as all the introns and exons of a gene of interest so that it can be expressed at physiological levels. This was done with the Hh protein Costal-2 (Cos2) (Zadorozny, Little et al. 2015). The *cos2* gene was cloned into an att vector that permitted the inclusion of the introns, 3'UTR, and promoter sequence so that the entire genomic sequence for wild type and mutants could be compared at a specific locus in the third chromosome.

This technique was also used in my research to investigate the transcription factor *ci* that I will discuss in Chapter 3 and 4.

*CRISPR/Cas9*

The CRISPR/Cas 9 gene editing technique is a tool that allows for a direct manipulation of DNA and it is particularly convenient to use in Drosophila. Cas9 is an endonuclease that cuts the DNA at a specific sequence determined by the position of PAM motifs and the corresponding guide RNAs. This causes a double stranded break that the cell can repair through one of two mechanisms: Nonhomologous End-Joining (NHEJ) or Homology Directed Repair (HDR). In the absence of a donor template, the cell will fuse the DNA back together inaccurately. In the presence of a repair template known as a donor template, the cell will use the information from that sequence to repair the DNA and incorporate the desired DNA alteration (Vervoort, Crozatier et al. 1999). This was the technique I used to create CRISPR *ci* alleles that I will discuss in Chapter 4.

*Flp/FRT Recombination: clonal analysis*

We use the Flp/FRT recombination system to create a homozygous recessive mutation or activate the expression of a transgene in only a subset of cells in the wing disc. The Flippase (Flp) recognizes the inverted FRT sites on specific sites on the chromosome and induces recombination in only a certain subset of cells (clone). The FRTs are located at the same location of two chromosomes near the centrosome so that recombination between two FRTs during mitosis leads to the creation of daughter cells that are homozygous for almost the entire chromosome arm distal to the FRT site.

This is preferable to studying the deletion/mutation in the whole disc because it generally permits recovery of cells with the desired genetics without killing animals prior to analysis and without distorting the overall wing disc development and morphology; clones also provide an internal control for measuring the effects of the genetic manipulation. There are two basic techniques to study this system: positive and negative marking.

Negative marking ubiquitously expresses GFP throughout the wing disc, upon heat shock the FLP recombinase induces recombination at the FRT sites which results in a clone without GFP, as well as a sister clone with two doses of GFP. Positive marking makes use of the UAS/Gal4 system that requires the expression of UAS-GFP, Gal4 and Gal80. Before heat shock, Gal80 is expressed throughout the disc and inhibits Gal4 so the UAS-GFP is off. After heat shock recombination, one daughter cell will have two copies Gal80 and the other daughter cell will not have Gal80, allowing for the expression of Gal4, UAS-GFP as well as any deletion or UAS driven gene/RNAi of interest (Figure 4). This method is beneficial because it restricts the expression of a UAS transgene to a specific subset of cells instead of throughout the wing disc which may compromise wing disc morphology.

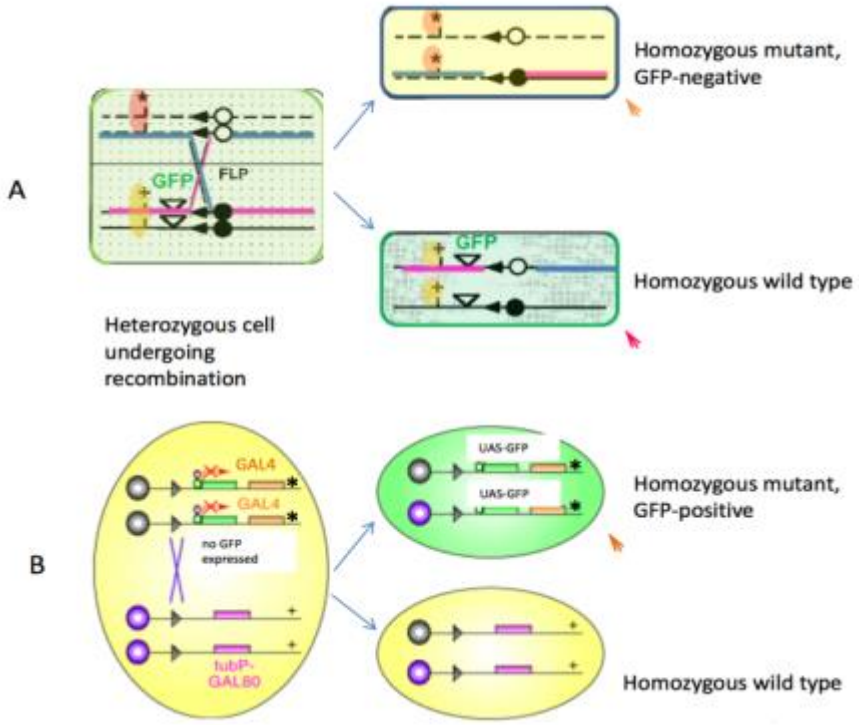



**Figure 4** Flp/FRT Recombination Diagram for Negative and Positive Marking: adapted from (Lee and Luo 2001) (Zadorozny 2014) (Theodosiou and Xu 1998) A) Negative marking: A parent cell is initially heterozygous for a mutation of interest and ubiquitously expresses GFP, after heat shock for 1 hour at 37 degrees, FLP recombinase is induced to initiate recombination in dividing cells. One daughter cell has two copies of the desired mutation and is absent of GFP while the other daughter cell (the twin spot) has two copies of GFP. B) Positive marking: A parent cell has tubP-Gal80, Gal4, UAS-GFP and a desired mutation. In the parent cell Gal80 inhibits Gal4 expression so the GFP is turned off. After heat shock, one daughter cell receives two copies of Gal80 while the other daughter cell has no Gal80, can express Gal4 and subsequent UAS-GFP as well as UAS driven transgenes.

We have used both positive and negative marking to understand how altered transgenes function to regulate Hh signaling. For example, we wanted to know if the binding of Fused

kinase (Fu) to Costal2 (Cos2) was important for regulating the processing of the Hh transcription factor Ci from its full-length Ci-155 form to the Ci-75 repressor. We tested this by creating UAS-Cos-Wt and UAS-Cos2ΔFu transgenes in *cos2* null positively marked clones where we could specifically express UAS-Cos-Wt in just the clone. Loss of Cos2 blocks processing and promotes elevated Ci-155 levels in the clone while expression of UAS-Cos-WT and UAS-CosΔFu rescues Ci-155 levels back to normal. This would suggest that Fu binding to Cos2 is not important for processing, but we also found that UAS-Cos was highly overexpressed and there might have been residual weak binding of Fu. So, we looked at physiologically expressed genomic Costal2 (gCos2)-Wt and gCos-ΔFu in negatively marked *cos2* clones which showed that there is elevated Ci-155 levels in the binding mutant compared to wild-type revealing that Fu binding to Cos2 is important for efficient Ci-155 processing as discussed in Chapter 2 (Figure 5).

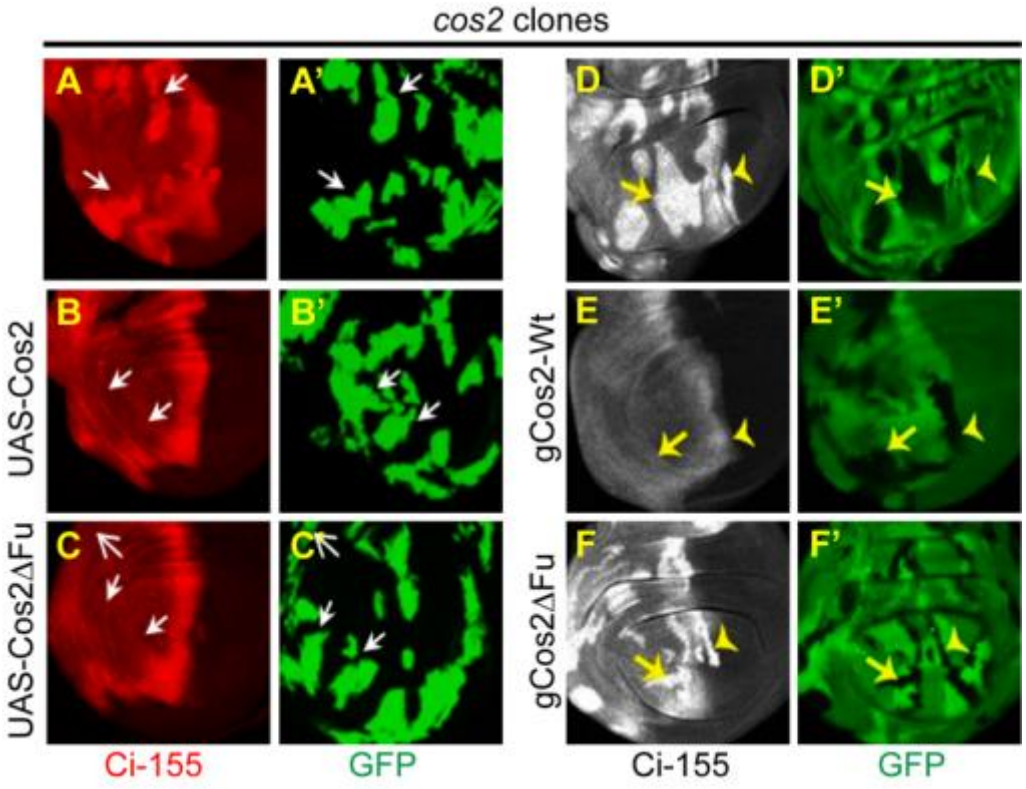
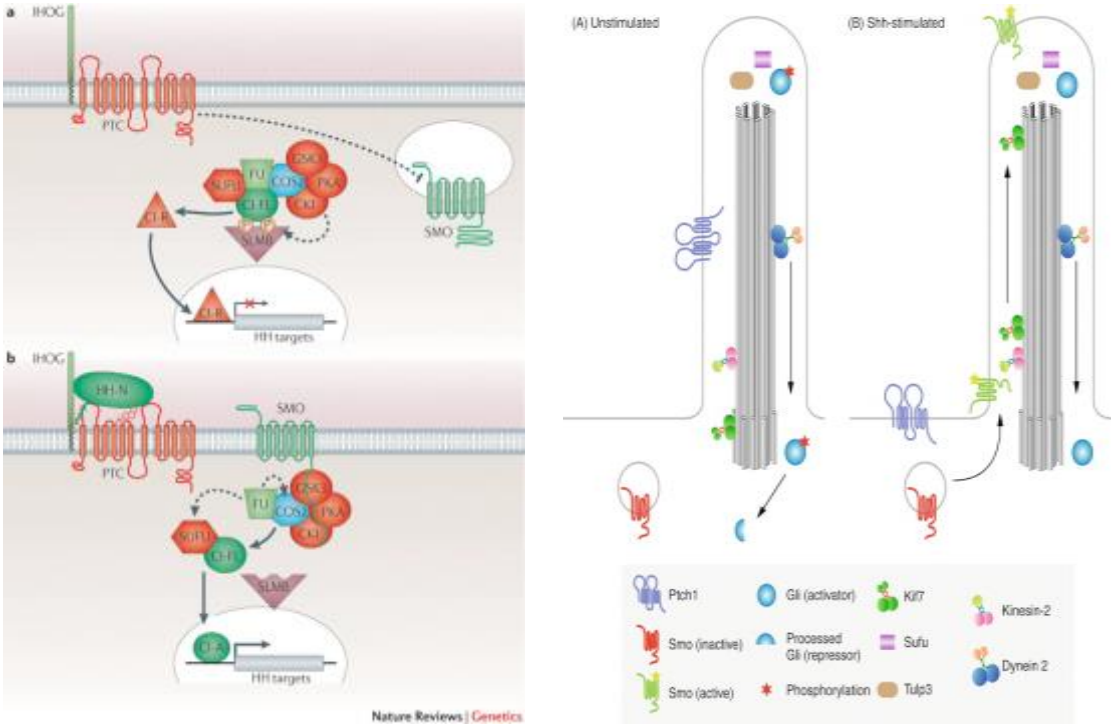



**Figure 5 Rescue of null Costal2 by UAS-Cos and gCos:** (A-C) Positively marked *cos2* clones are marked by GFP in the clones where we measured Ci-155 levels. (A-A') Loss of Cos2 stimulates elevated Ci-155 (red) levels due to lack of processing. (B-B') UAS-Cos2-Wt was expressed in just the GFP clone (not throughout the whole disc) and reduced Ci-155 levels compared to the *cos2* clones, indicating rescue. (C-C') UAS-CosΔFu binding mutant was expressed in the GFP positively marked clones, where Ci-155 levels were reduced compared to *cos2* clones alone, indicating rescue. (D-F) Negatively marked clones where the absence of GFP indicates the loss of Cos2. (D-D') Loss of Cos2 elevated Ci-155 (gray) levels in the anterior and at the AP border. (E-E') gCos-Wt was expressed at physiological levels throughout the fly and wing, where *cos2* clones were induced but did not show elevated Ci-155. (F-F') gCosΔFu was

expressed throughout the fly and wing, where *cos2* clones were induced which did show elevated Ci-155 levels, indicating that binding of Fu to Cos2 is important for processing.

**Overview of Hh Signaling**




A) B)

**Figure 6 Hh Signaling in Drosophila vs Mammals:** A) adapted from (Ingham, Nakano et al. 2011) Flies: In the absence of Hh signaling, the receptor Patched (Ptc) actively inhibits the actions of another transmembrane protein Smoothened (Smo), which is kept at low levels mainly bound to internal vesicles. Cos2 is bound to Fused (Fu) protein and acts a protein scaffold for kinases Protein Kinase A (PKA), Glycogen Synthase-3 (GSK3), and Casein Kinase-1 (CK1) to come together and phosphorylate the full-length transcription factor, Ci-155. Phosphorylated Ci-155 creates a binding site for Slimb, as the substrate recognition component of the SCF Cul1 E3 ubiquitin ligase complex, this promotes the partial proteolysis of full length Ci-155 to its shortened N-terminus repressor form Ci-75. Suppressor of Fused (Su(fu)) restrains the remaining

Ci-155 in the cytoplasm. In the presence of Hh, Hh binds to its receptor Ptc, which is then degraded. Smo is activated by PKA and CK1 phosphorylation that cause the disassembly of the Cos2 kinase complex with Ci and the activation of Fu, which is necessary for the activation of Ci-155 (Ingham, Nakano et al. 2011). B) (adapted from Goetz, Ocbina et al. 2009). Mammals: In the absence of Shh, mammalian Patched prevents the accumulation of Smo in the primary cilium. Kif7, the mammalian Cos homologue, acts as a protein scaffold for PKA, GSK3 and CK1 causing the mammalian Hh transcription factors Gli 2/3 (Ci homologues) to be processed into their repressor forms. Su(fu) restrains Gli activity at the tip of the cilium. In the presence of Shh, Ptch1 allows for the activation and accumulation of Smo in the primary cilium. Kif 7 migrates to the tip and along with Su(fu), allows for the release and activation of the Gli proteins (Goetz, Ocbina et al. 2009).

***Hedgehog***

In a Hh producing cell, Hh is initially translated into a 45kD protein in the endoplasmic reticulum (Lee, Ekker et al. 1994). This protein complex undergoes autoproteolysis to form the N terminus Hh (Hh-N) and the C terminus Hh (Hh-C) (Chen, Tukachinsky et al. 2011). Hh-N then undergoes two lipidation steps: 1) Hh-C, a transient cholesterol transferase, adds cholesterol and 2) Skinny Hedgehog (ski), an acyl transferase, adds fatty acids to Hh-N through a process called palmitoylation (Pepinsky, Zeng et al. 1998).

Lipidated Hh is endocytosed on the apical membrane and transported to the basolateral surface of the cell; it is then secreted through the actions of cytonemes and transmembrane protein, Dispatched (Disp) (Callejo, Bilioni et al. 2011) (Burke, Nellen et al. 1999). Cytonemes are actin based cellular extensions that assist in carrying signaling molecules; they were

identified in the Drosophila wing disc and support transport of the Hh protein (Ramirez-Weber and Kornberg 1999). Basal cytonemes in posterior cells facilitate the trafficking of Hh to the anterior; modification to cytoneme formation affects the overall Hh gradient length (Bischoff, Gradilla et al. 2013).

Hh travels both short and long range distances of up to fifty micrometers in the Drosophila wing disc (Zhu and Scott 2004). This is assisted by glycosylphosphatidylinositol-linked heparan sulfate proteoglycans (HSPG), Dally and Dally-like (Han, Belenkaya et al. 2004). Dally and Dally like are the substrates of Tout-velu (Ttv), a heparin sulphate polymerase, which is necessary for Hh diffusion and migration from the posterior to the anterior in the Drosophila wing disc (Bellaiche, The et al. 1998). Once the Hh ligand reaches its cellular destination, the glycosyl-phosphatidyl-inositol (GPI anchor) of Dally-like stimulates the internalization of Hh bound to the Patched receptor, permitting activation of the Hh signaling pathway (Gallet, Staccini-Lavenant et al. 2008).

*Patched (Ptc)*

In a Hh receiving cell, Ptc is a 12-pass transmembrane protein and is the main receptor of the Hh ligand in Drosophila (Ingham, Taylor et al. 1991). Ptc works with two functionally redundant single pass membrane proteins called Interference Hedgehog (Ihog) and Brother of interference Hedgehog (Boi) (Yan, Wu et al. 2010). Collectively, these receptors prevent the further mobility of Hh by promoting the endocytosis and subsequent degradation of the ligand (Torroja, Gorfinkiel et al. 2004). Ihog predominantly functions to bind to the extracellular Hh and can synergize with Ptc for better binding to Hh (Zheng, Mann et al. 2010).

In vertebrates Patched1/2 (Ptch1/2) functions as the main receptor in partner with Cdo and Boc, Ihog and Boi's mammalian homologues (Carpenter, Stone et al. 1998) (Tenzen, Allen et al. 2006). In contrast to Drosophila, Cdo and Boc have altered binding modes and contacts to Shh; they do not synergize with Ptch1 to enhance binding to their ligand (McLellan, Zheng et al. 2008). Ihog/Boi and Cdo/Boc function redundantly, when both of the co-receptors cannot function there is a loss of downstream Hh activity (Allen, Song et al. 2011). In comparison, loss of Ptc/Patch1 causes an increase in Hh signaling due to the lack of inhibition of Smo (Alcedo, Ayzenzon et al. 1996).

*Smoothened*

Smo is a Frizzled Class (Class F) G-protein coupled receptor-like (GPCR-like) heptahelical transmembrane (7TM) protein, which is activated in response to Hh (van den Heuvel and Ingham 1996) (Alcedo, Ayzenzon et al. 1996) (Isberg, de Graaf et al. 2015). Smo is structurally similar to GPCRs because 1) it has a conserved N-terminal cysteine rich domain (CRD) that can bind to sterols (Nachtergaele, Whalen et al. 2013) (Rana, Carroll et al. 2013), 2) it has intracellular loops (IC) that can signal through heterotrimeric G-protein Galpha (Ogden, Fei et al. 2008), and 3) it has a carboxy tail that is phosphorylated in part by a GPCR kinase (gprk-2) (Molnar, Holguin et al. 2007) (Chen, Li et al. 2010). Together the CRD, IC, and the carboxy tail regulate Smo to promote the downstream activation of Hh target genes.

Unlike typical GPCRs, the Hh ligand doesn't directly bind Smo; Hh binds to Ptc, which indirectly causes a Smo conformational change through a mechanism that is not well understood (Arensdorf, Marada et al. 2016). Ptc is similar to the resistance, nodulation, division (RND) family of bacterial proton gradient-driven transmembrane molecular transporters, and could

indirectly inhibit Smo activity by acting as a small-molecule pump (Taipale, Cooper et al. 2002). In mammals, there are two binding sites on SMO that can be targeted for potential drug agonists/antagonists: 1) the binding region which cyclopamine can bind to inhibit Smo function and 2) the binding region which sterols bind to activate Smo (Wang, Wu et al. 2014). Sterols bind to Smo CRD region but it was unclear what the critical endogenous activator was until a recent series of structural studies identified it to be cholesterol (Byrne, Sircar et al. 2016) (Huang, Nedelcu et al. 2016). Therefore, it is thought that Ptc may regulate the exposure of Smo to cholesterol, but direct evidence for this theory remains elusive.

In flies Hh signaling induces the upregulation of Smo, causing Smo to compete with Ci-155 as a phosphorylation substrate for PKA (Ranieri, Therond et al. 2014). Smo is stimulated by the hyperphosphorylation of its carboxyl tail by kinases CK1, Gprk-2, and PKA to promote different intensities of the Hh morphogenic response (Jia, Tong et al. 2004) (Li, Ma et al. 2014). Phosphorylated Smo interacts with downstream binding partner Cos2 and PKA to form an active signaling complex that prevents Ci processing and promotes Ci activation (Zhang, Zhao et al. 2005). Only in flies, does Smo have a large C-terminal tail with PKA/CK1 sites but in both vertebrates and flies, Gprk-2/GRK2 phosphorylate the carboxyl terminal tail to promote the translocation of Smo into the primary cilium where it migrates and becomes enriched at the ciliary tip and induces the activation of Gli proteins (Milenkovic, Weiss et al. 2015) (Chen, Yue et al. 2011).

**Regulation of Cubitus Interruptus (Ci)**

Ci was discovered previous to Hh signaling when researchers found a recessive mutant gene on the fourth chromosome that caused aberrations in the fourth longitudinal wing vein (cubitus) (Tiniakow and Terentieva 1933). Further research found that Ci is a Zn finger transcription factor and is highly homologous to the human zinc finger protein Gli, which is overexpressed in gliobastomas (Orenic, Slusarski et al. 1990). The first connection between Hh and Ci was identified when it was discovered that in mutant *fused* and *hh* flies, the distribution of the Ci protein was altered (Motzny and Holmgren 1995). Functionally, Ci was found to be essential for transducing the Hh signal to facilitate patterning and limb development (Dominguez, Brunner et al. 1996). Hh signaling promotes the activation of full-length Ci-155 which enters the nucleus and induces the transcription of Hh target genes (Ohlmeyer and Kalderon 1998). In the absence of the Hh signal, Ci-155 is processed to a Ci-75 repressor form which enters the nucleus and inhibits the transcription of Hh specific target genes (Aza-Blanc, Ramirez-Weber et al. 1997). Current research focuses on Ci regulation by investigating how Hh promotes Ci processing, degradation, and activation.

*Studying Ci in Drosophila Wing Discs*

*ci* is expressed throughout the anterior of the fly wing disc where Ci-155 levels are readily visualized by the 2A1 antibody which recognizes the C-terminus of full length Ci. However, when UAS-Ci variants are studied with a C765 Gal4 driver, UAS-Ci is ectopically expressed in the posterior as well as the anterior at rather high levels and typically co-expressed with endogenous Ci. To discern the effects of the UAS-Ci variant from endogenous Ci, the UAS-Ci transgenes can have a Myc tag in the C-terminus to monitor the effects of Ci levels in just the

variant. For example, we found that there was enhanced processing in UAS-CiΔCORD because Myc tag had a narrower Ci stripe at the AP border than in wild-type (Zhou and Kalderon, 2010).

Unfortunately, monitoring the levels of Ci-75 repressor has been a great challenge for Hh signaling. There used to be an antibody against the Zn finger domain that could recognize both the Ci-75 repressor and full length Ci-155 at the N-terminus, which was visualized in a western blot that showed a band at 155kd and 75kd (Ohlmeyer and Kalderon 1998). Currently our lab and other labs who study Ci processing don't routinely use this antibody because of issues with repeatability and doubts that antibody still works, I personally have also tried and was unable to get a signal. Therefore, most labs routinely study Ci-75 repressor formation by using the *hh-lacZ* assay which detects small amounts of repressor.

In a *hh-lacZ* experiment, we induce *smo* clones in a wing disc where UAS-Ci is ectopically expressed in the posterior and stain for *hh-lacZ* expression. *smo* clones block Hh signaling and promote the expression of Ci-75; since *hh* is a target of Ci-75 repressor, there will be a reduction of *hh-lacZ* in the clone. For example, to determine that a *cos2* mutant has blocked processing, you would perform the *hh-lacZ* assay for a *smo cos* mutant clone and not expect any reduction in *hh-lacZ* expression within the clone.

*Costal Regulation of Ci*

Costal 2 (Cos2) is a kinesin-like protein that can bind to microtubules; it was originally identified in Drosophila to regulate Ci activity (Sisson, Ho et al. 1997) (Robbins, Nybakken et al. 1997). Two mammalian proteins, Kif7 and Kif27, were identified based on sequence similarity to Cos2; they were initially determined to have a non-essential role in Hh signaling that was compensated by the structure of the primary cilium (Varjosalo, Li et al. 2006). Further research

however found that Kif7 was able to promote processing of Gli2 and Gli3 while Cos was functionally able to substitute for Kif7 in Zebrafish (Maurya, Ben et al. 2013) (Cheung, Zhang et al. 2009).

Cos2 moderates both the positive and negative actions of Ci. Without Hh, Cos acts as a scaffold for kinases PKA, GSK3, and CK1 that are necessary for Ci partial proteolysis (processing) (Zhang, Zhao et al. 2005). These kinases induces a phosphorylation cascade that creates a binding site on Ci for Slimb, a member of the SCF (Skp1, Cul1, Roc1/Rbx RING finger protein and substrate recognition F-box protein) family of E3 ubiquitin ligases; Slimb promotes Ci processing from full length Ci-155 to Ci-75 repressor (Aza-Blanc, Ramirez-Weber et al. 1997) (Jiang and Struhl 1998). Ci-155 and Ci-75 have opposing transcriptional effects on the regulation of Hh target gene induction. For example, *dpp* is induced by full length Ci-155 while Ci-75 repressor will actively bind to the same DNA sequence to restrict *dpp* transcription (Muller and Basler 2000).

Fu and PKA are serine/threonine protein kinases associated with Cos2 in Drosophila that support Ci processing and activation (Preat, Therond et al. 1990). Loss of Cos2 or PKA in the fly wing discs completely blocks Ci processing, allowing for the ectopic accumulation of Ci-155 and increased Ci activity independent of Hh regulation (Price and Kalderon 1999) (Wang, Wang et al. 1999) (Ohlmeyer and Kalderon 1997) (Li, Ohlmeyer et al. 1995) (Jiang and Struhl 1995). When Fu cannot bind to Cos2, Fu is destabilized and Ci processing is impaired (Zadorozny, Little et al. 2015). We hypothesize that Cos2 has an additional role to silence Ci-155 activity in the absence of processing by binding to the Ci-155 CORD region, which I will discuss in Chapter 4.

Cos2 supports Hh signaling by stimulating Smo accumulation at the membrane as well as promoting Ci activation in response to high levels of Hh signaling (Zhao, Tong et al. 2007). Phosphorylated Smo stimulates the translocation of Cos/Fu to the membrane which induces Fu dimerization and auto-phosphorylation of its activation loop by Fu and CK1 (Shi, Li et al. 2011) (Zhou and Kalderon 2011) (Zhang, Mao et al. 2011). Fu binding to Cos2 is necessary for this dimerization and cross-phosphorylation, stimulating complete Fu activation necessary for Ci activity (Zadorozny, Little et al. 2015).

*Roadkill Regulation of Ci*

Hib also known as Roadkill (rdx) is induced by Hh signaling at the AP Border to promote complete Ci-155 proteolysis. Hib is a BTB protein (Broad Complex, Tramtrack, and Bric a Brac) which is the substrate recognition component for Cul3 E3 ubiquitin ligase complex to facilitate the degradation of Ci-155 (Zhang, Zhang et al. 2006). This proteolysis mechanism differs from Ci-155 partial proteolysis by Slimb, the substrate recognition component for Cul1 E3 SCF ubiquitin ligase complex. The SCF (Skp, Cul-1, F-box) family and Cul3 based complexes are both multi-protein complexes that recognize their substrates and promote their subsequent degradation; the major difference is the mode of recognition in which Cul1 uses an F-box protein (Slimb) and Cul3 uses BTB protein (Hib) (Pintard, Willems et al. 2004).

At the AP Border, Hib downregulates Su(fu) through the spliceosome factor Crooked Neck (Crn) (Seong and Ishii 2013) (Liu, Zhou et al. 2014). Loss of Su(fu)/Ci binding causes Ci-155 destabilization in which there are lower Ci-155 levels throughout the anterior of the wing disc (Ohlmeyer and Kalderon 1998). Since Hib is not present in the far anterior of the wing disc, Ci-155 stability dependent on Su(fu) is mediated by a separate mechanism. It is unclear to what

extent Hh regulates Su(fu)-modulated proteolysis and Hib-dependent proteolysis to facilitate complete Ci degradation, thus we will investigate each mechanism independently by using Ci transgenes that block Roadkill or Su(fu) Binding discussed in Chapter 4.

*Su(fu) Regulation of Ci*

Su(fu) was first identified in Drosophila by its ability to rescue the loss of Fu kinase mutant, $fu^{mH63}$, in adult wings and the segment polarity phenotype caused by $fu^{1}$ mutant in Drosophila embryos (Preat 1992). Fu mutants cause a reduction in the expression of *ptc-lacZ* at the AP border of developing larval wing discs that can be rescued by the additional loss of Su(fu), but the highest levels activity is not restored illustrated by the continued loss of Engrailed (Zhou and Kalderon 2011). Loss of Su(fu) alone has no obvious phenotype on Hh signaling activity or adult viability but enhances Ci-155 proteolysis throughout the larval wing disc by a mechanism independent of Slimb mediated Ci-155 processing (Ohlmeyer and Kalderon 1998). How Su(fu) stabilizes Ci-155 levels and inhibits Ci-155 activity is an area of active research that we are investigating in Chapter 4.

Mammalian SUFU is highly conserved to Drosophila Su(fu); human SUFU was cloned and found to interact with Gli1 to inhibit its transcriptional activity (Kogerman, Grimm et al. 1999). Even though Su(fu) is relatively dispensable in flies, loss of SUFU in mice results in embryonic lethality (Svard, Heby-Henricson et al. 2006). SUFU inhibits Gli by restraining the activity of Gli2/3 and by promoting the processing of Gli3 to its repressor forms (Wang and Li 2006) (Humke, Dorn et al. 2010). Hh stimulates the rapid dissociation of the SUFU-Gli complex and promotes Gli nuclear translocation (Tukachinsky, Lopez et al. 2010). Recent studies have also shown a positive role for SUFU in Hh signaling, requiring SUFU for maximum Hh

signaling by binding to Gli1 and promoting its nuclear accumulation in the nucleus while simultaneous promoting the nuclear export of Gli3 repressor (Zhang, Shen et al. 2017)

*Fu Regulation of Ci*

*fu* mutant flies were identified because they had altered wing vein patterning in veins 3 and 4 (Fausto-Sterling 1978). Based on sequence similarity studies, it was found that *fu* encodes for serine/threonine kinase (Preat, Therond et al. 1990). In Drosophila, Fu is necessary for Ci activity and functions primarily to antagonize Su(fu) but this is not conserved in mammals. Mouse FUSED (FU) does not appear to be necessary for Hh signaling, but is important for survival; loss of FU in mice resulted in death by postnatal day 21 due to defects in motile cilia (Wilson, Nguyen et al. 2009).

Hh signaling stimulates Fu auto-activation through the actions of Smo/Cos2 causing Fu to aggregate at the membrane (Zhou and Kalderon 2011). Fu forms a trimeric complex with Ci and Cos2 as well as a tetrameric complex with Ci, Cos2, and Su(fu) (Stegman, Vallance et al. 2000). However, it is not clear whether Fu directly binds Ci nor is it known how Fu causes the activation of full length Ci-155. Fu phosphorylates Su(fu) at sites S321 and S324 but this does not appear to be functionally relevant for Ci activation (Zhou and Kalderon 2011). At higher levels of Hh signaling, Fu phosphorylates Cos2 at site S973; at moderate levels of Hh signaling, Fu phosphorylates Cos2 at site S572. Yet mutation of these sites to alanine did not affect Ci activity in the wing disc (Zadorozny, Little et al. 2015) (Ranieri, Ruel et al. 2012) (Ho, Suyama et al. 2005). We thought that perhaps Su(fu) and Cos2 served redundant functions but when we mutated both potential target sites on these proteins, the wings discs had no change in Ci activity.

Therefore, the critical target site of Fu is not known and we continued to investigate the mechanism of Ci activation by Fu in Chapter 4.

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# Chapter 2

Contributions of Costal2-Fu Interactions to Hedgehog Signaling in Drosophila: This was a collaborative effort between Eva, Dan, and me. Eva cloned gCostal2 and the variants, maintained the fly stocks, and did experiments for Figure 1F-K and Figure 2A-F. I did the dissections, clonal analysis, and immunostaining for the other figures in the paper. Dan maintained the fly stocks and set up most of the crosses.

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RESEARCH ARTICLE

# Contributions of Costal 2-Fused interactions to Hedgehog signaling in *Drosophila*

**Eva V. Zadorozny*, Jamie C. Little* and Daniel Kalderon‡**


## ABSTRACT

The *Drosophila* kinesin-family protein Costal 2 (Cos2) and its mammalian ortholog Kif7 play dual roles in Hedgehog (Hh) signaling. In the absence of Hh, Cos2 and Kif7 contribute to proteolytic processing and silencing of the Hh-regulated transcription factors, *Drosophila* Cubitus interruptus (Ci) and mammalian Gli proteins. Cos2 and Kif7 are also necessary for full activation of full-length Ci-155 and Gli transcription factors in response to Hh proteins. Here, we use classical *fused* alleles and transgenic Cos2 products deficient for Fused (Fu) association to show that Cos2 must bind to Fu to support efficient Ci-155 processing. Residual Ci-155 processing in the absence of Cos2-Fu interaction did not require Suppressor of Fused, which has been implicated in processing mammalian Gli proteins. We also provide evidence that Cos2 binding to the CORD domain of Ci-155 contributes to both Ci-155 processing and Ci-155 silencing in the absence of Hh. In the presence of Hh, Ci-155 processing is blocked and Cos2 now promotes activation of Ci-155, which requires Fu kinase activity. Here, we show that normal Ci-155 activation by Hh requires Cos2 binding to Fu, supporting the hypothesis that Cos2 mediates the apposition of Fu molecules suitable for cross-phosphorylation and consequent full activation of Fu kinase. We also find that phosphorylation of Cos2 by Fu at two previously mapped sites, S572 and S931, which is thought to mediate Ci-155 activation, is not required for normal activation of Ci-155 by Hh or by activated Fu.


**KEY WORDS: *Drosophila*, Hedgehog, Signal Transduction, Costal 2 (Cos), Fused**

## INTRODUCTION

Secreted Hedgehog proteins constitute a major family of signaling molecules that guide development, cell proliferation and stem cell behavior in *Drosophila* and in mammals (Hui and Angers, 2011; Briscoe and Therond, 2013; Zhang and Kalderon, 2001; Peng et al., 2013; Petrova et al., 2013; Li et al., 2014). Accordingly, genetic alterations affecting Hedgehog (Hh) signaling are responsible for a variety of developmental defects and cancers, prompting the development of promising therapeutic drugs (Ng and Curran, 2011; Metcalfe and de Sauvage, 2011; Amakye et al., 2013).

The majority of responses to Hh signals are transcriptional changes mediated by the zinc-finger DNA-binding protein Ci in *Drosophila* and a family of three orthologs, Gli1, Gli2 and Gli3, in mammals (Hui and Angers, 2011; Briscoe and Therond, 2013). Full-length Ci-155, like Gli2 and Gli3, is processed by the proteasome to a C-terminally truncated repressor (Ci-75) in the absence of Hh. Proteolytic processing depends on prior phosphorylation of Ci-155 at a cluster of PKA, CK1 and GSK3 sites, which are conserved in Gli2 and Gli3, and on recognition of those phosphorylated residues by a conserved Cul1-containing E3 ubiquitin ligase. Processing also involves a kinesin-family molecule, Costal 2 (Cos2; Cos – FlyBase), or Kif7 in mammals, which binds to Ci-155 or Gli2/3. In *Drosophila*, Cos2 also binds to PKA, CK1 and GSK3, thereby acting as a scaffold to enhance Ci-155 phosphorylation (Zhang et al., 2005). When Hh binds to Patched (Ptc) and to its co-receptor, Smoothened (Smo) is activated (Hui and Angers, 2011; Briscoe and Therond, 2013). Activated Smo leads to inhibition of Ci/Gli protein processing and activation of full-length Ci/Gli proteins by mechanisms that include gaining increased nuclear access and, likely, dissociation from the inhibitory proteins Cos2/Kif7 and Suppressor of Fused (Hui and Angers, 2011; Briscoe and Therond, 2013). Activation of Ci-155 depends substantially on Fu protein kinase activity in *Drosophila*, while the protein most similar to Fu in mice has no role in Hh signaling (Alves et al., 1998; Ohlmeyer and Kalderon, 1998; Wilson et al., 2009; Zhou and Kalderon, 2011).

Hh signaling has been studied extensively in developing *Drosophila* wing imaginal discs (Ingham and McMahon, 2001). Here, Hh expression is confined to posterior compartment cells, whereas Ci is expressed only in anterior compartment cells. Hh therefore signals in a graded fashion to anterior cells in a central domain of 12-15 cells' width, known as the AP (anterior/posterior) border. Ci-155 processing is substantially inhibited throughout the AP border and the target gene *decapentaplegic* (*dpp*) is transcriptionally induced through most of this region (Methot and Basler, 1999). Induction of *ptc* transcription, which is commonly visualized with a *ptc-lacZ* reporter gene, is restricted to the posterior half of this signaling domain, whereas Engrailed (En) is induced only very close to posterior Hh-secreting cells (Vervoort, 2000). Hh signaling has also been studied biochemically *in vitro* and in tissue culture to define and assess the role of specific protein interactions and modifications, but these inferences are limited by the appreciation that normal Hh signaling depends on maintaining the normal stoichiometry of key signaling components, including Cos2. Here, we investigated the roles of Cos2 binding to Fu and to nucleotides, and the role of Fu phosphorylation sites on Cos2 under physiological conditions.

## RESULTS

### Fused C-terminal Cos2-binding domain is required for efficient Ci-155 processing

Prior studies have shown that C-terminal truncations of the Fu protein affect Ci-155 processing but there are conflicting claims regarding whether Fu is essential for Ci-155 processing and whether some *fu* alleles simply make Ci-155 processing more sensitive to Hh inhibition (Alves et al., 1998; Wang and Holmgren, 1999; Methot and Basler, 2000; Lefers et al., 2001). Wing discs from male third instar larvae hemizygous for $fu^{M1}$ (encoding only residues 1-80 of the normal Fu protein), $fu^{W3}$ (encoding residues 1-612) and $fu^{RX2}$

Department of Biological Sciences, Columbia University, New York, NY 10027, USA.
*These authors contributed equally to this work

‡Author for correspondence (ddk1@columbia.edu)

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(encoding residues 1-748) (Therond et al., 1996) (Fig. 1A) all exhibited increased Ci-155 levels throughout the anterior compartment that were highest in the broadened AP domain of Hh signaling, suggesting ubiquitously impaired Ci-155 processing that is inhibited further by Hh (Fig. 1B-E). A strong cell-autonomous increase in Ci-155 staining was seen in homozygous *fu* mutant clones for all three alleles in regions beyond the range of Hh and also in anterior *smo* $fu^{M1}$ clones (Fig. 1J; supplementary material Fig. S1A-C), showing a strong Ci-155 processing defect in the absence of any response to Hh.

We then explored the properties of the $fu^{M1}$ allele, which is closest to a null, in more detail. The levels of C-terminally tagged Ci from GAL4-driven *UAS-Ci-Myc* transgenes can report Ci-155 stability because transgene transcription is expected to be constant. Thus, Ci-Myc expressed evenly throughout the wing disc is present at lower levels in anterior cells than in AP border or posterior cells because processing in anterior cells eliminates the Myc epitope (Zhou and Kalderon, 2010). This difference is still greater for the Ci variant CiΔCDNΔCORD (henceforth abbreviated as Ci-ΔΔ) (Fig. 1F,H), which appears to be processed more efficiently than wild-type Ci (Zhou and Kalderon, 2010). In $fu^{M1}$ mutant discs, Myc staining of both wild-type Ci-Myc and CiΔΔ-Myc was increased in anterior cells to almost the same level as the AP border, confirming impaired processing in the absence of Hh (Fig. 1F-I).

We then used an assay that can detect even low levels of Ci-155 processing (Methot and Basler, 2000; Price and Kalderon, 2002; Smelkinson et al., 2007). In this assay, a Ci transgene is expressed throughout the wing disc (using *C765-GAL4+UAS-Ci* transgenes) and conversion to the Ci-75 repressor is assayed in posterior *smo* mutant clones (to block Hh signaling) by examining transcriptional repression of a *hh-lacZ* reporter. No Ci-155 processing is detected by this *hh-lacZ* repression assay in the absence of Cos2 function in *smo cos2* mutant clones (supplementary material Fig. S2A) (Methot and Basler, 2000; Zhou and Kalderon, 2010). However, sufficient Ci-75 was produced in *smo* $fu^{M1}$ clones to give strong repression of the *hh-lacZ* reporter (Fig. 1L), in contrast to previously reported results for $fu^{A}$ (Methot and Basler, 2000). We found that $fu^{A}$ mutant wing discs have the highest levels of Ci-155 in the AP border region (supplementary material Fig. S1D,E), as seen for the other three *fu* alleles tested, suggesting that some Ci-155 processing persists in the absence of Hh. We therefore suggest that there is residual Ci-155 processing for all *fu* alleles.

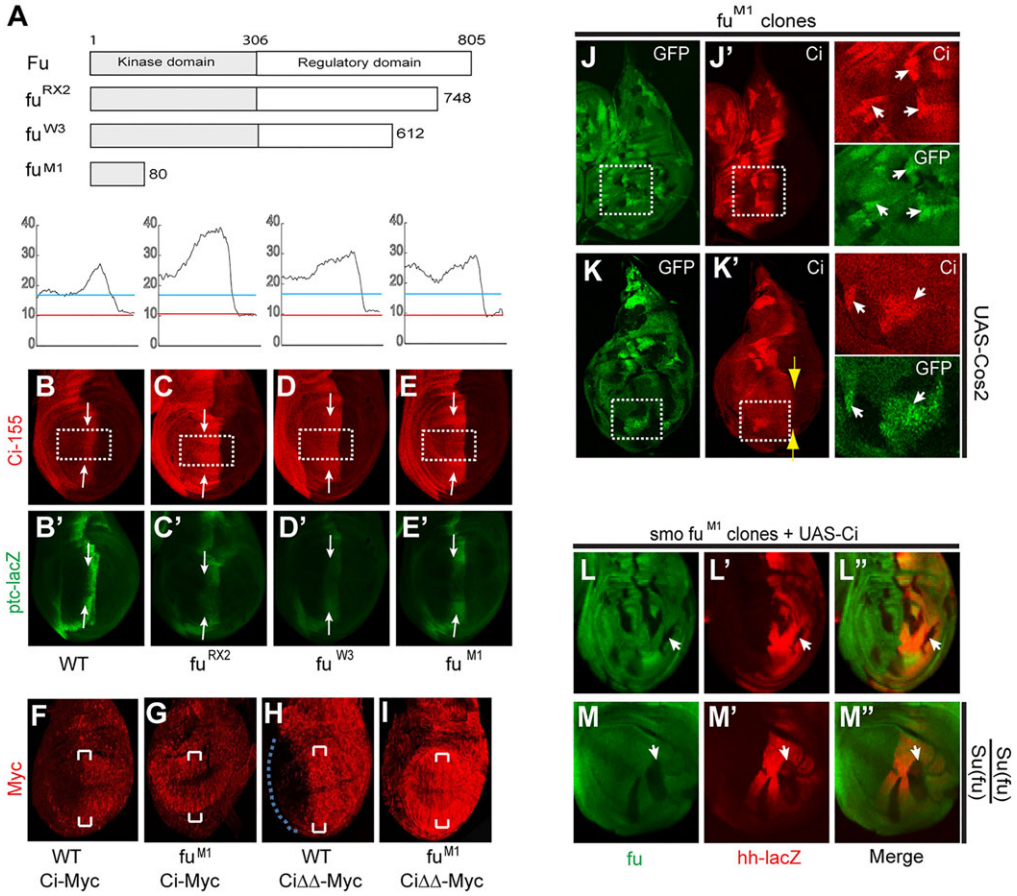


**Fig. 1. The C-terminal of Fused is required for efficient Ci-155 processing.** (A) Schematic of proteins encoded by *fused* alleles used. (B-E) Full-length Ci-155 (red) and (B′-E′) *ptc-lacZ* reporter of Ci activity (green) in wing discs from male wild-type (WT) or *fu* mutant larvae. Arrows indicate the anterior (left) boundary of *ptc-lacZ* expression. Plots of Ci-155 staining intensity (above B-E) along the AP axis were generated as described in the Materials and methods for the boxed regions. Background posterior Ci-155 levels (red line) and anterior Ci-155 levels (blue line) in wild-type discs are indicated. (F-I) Myc epitope staining (red) of Ci transgenes tagged with Myc at the C terminus and expressed ubiquitously using the *C765-GAL4* driver in wild-type (WT) or $fu^{M1}$ mutant wing discs. Brackets indicate the estimated AP border territory from parallel *ptc-lacZ* staining (not shown). (J-K′) Ci-155 levels (red) were increased in anterior $fu^{M1}$ clones (white arrows) marked by two copies of a *ubi-GFP* transgene (adjacent to GFP-negative twin-spot clones) in otherwise normal discs (J,J′) and in discs expressing *UAS-cos2* at a high level with the *C765-GAL4* driver (K,K′). Excess Cos2 inhibited Hh signaling and reduced Ci-155 staining at the AP border (yellow arrows). Insets show boxed regions at higher magnification. (L-M″) *hh-lacZ* (red) was repressed by Ci-75 repressor derived from *UAS-Ci* expressed using *C765-GAL4* in posterior $fu^{M1}$ $smo^{2}$ clones (arrows), marked by loss of Fu staining (green) in otherwise normal discs (L-L″) and in homozygous $Su(fu)^{LP}$ (null) discs (M-M″).

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We then tested whether excess Cos2 or Su(fu) can compensate for the absence of functional Fu. Excess Cos2 alone does not impair Ci-155 processing and can, in fact, reduce anterior Ci-155 below normal levels (Zhou and Kalderon, 2010). However, Ci-155 levels were still strongly elevated in $fu^{M1}$ clones in discs expressing high levels of a wild-type *cos2* cDNA (Fig. 1K). We also saw strong *hh-lacZ* repression in posterior *smo* $fu^{M1}$ mutant wing disc clones in the complete absence of Su(fu) (Fig. 1M). Thus, residual Ci-155 processing in the absence of functional Fu does not depend on Su(fu) and cannot be restored to normal by excess Cos2. All of the Fu variants that we have shown to have a similar deficit in Ci-155 processing (including Fu 1-748 encoded by $fu^{RX2}$) lack a C-terminal Cos2-binding domain (Robbins et al., 1997; Monnier et al., 2002), suggesting that Fu must bind to Cos2 to support efficient Ci-155 processing.

### Contribution of the Fu-binding domain of Cos2 to Ci-155 processing

We wished to test further whether Cos2 must recruit Fu to the Ci-155 processing complex by making Cos2 variants deficient for Fu binding. Previous studies identified a very small region of Cos2 (residues 543-605) sufficient for binding the regulatory (non-kinase) domain of Fu (Ruel et al., 2007). We therefore tested whether variants of Cos2 lacking this region could bind Fu by expressing HA-tagged Fu together with Flag-tagged Cos2 proteins in Kc tissue culture cells and measuring co-precipitation of HA-Fu with Flag-Cos2. Flag-Cos2 lacking residues 543-605 was not apparently stable, whereas Flag-Cos2 proteins lacking residues 560-580 or 580-600 co-precipitated HA-Fu efficiently (data not shown). However, Flag-Cos2 lacking residues 540-560 was stable and brought down very little HA-Fu compared with wild-type Flag-Cos2 (less than 1%; Fig. 2A). Although equal amounts of expression constructs were transfected in these experiments, extracts containing wild-type Flag-Cos2 contained far more HA-Fu than extracts from cells with Flag-Cos2Δ540-560 or no transfected Cos2 (Fig. 2A), reflecting previous observations that Cos2 strongly stabilizes Fu protein (Lum et al., 2003; Ruel et al., 2003). Thus, both HA-Fu levels and co-precipitation results indicate that Cos2 lacking residues 540-560 is highly deficient for Fu binding and we henceforth refer to that variant as CosΔFu.

We then examined the properties of CosΔFu in wing discs by expressing GAL4-responsive *UAS-CosΔFu* using the *C765-GAL4* driver but restricting expression to clones homozygous for the null $cos2^2$ allele by using the MARCM technique (Lee and Luo, 2001). Fu levels are greatly reduced in *cos2* mutant clones (Lum et al., 2003). However, expression of *UAS-Cos2* in *cos2* mutant clones increased the levels of Fu significantly beyond those in surrounding tissue (Fig. 2B). The level of Cos2 in those clones greatly exceeded endogenous levels (Fig. 2E) and presumably accounts for the observed increase in stable Fu protein. Although CosΔFu from *UAS-CosΔFu* was also present in great excess over endogenous levels in *cos2* mutant clones (Fig. 2F), Fu protein levels were much lower in these clones than in surrounding tissue (Fig. 2C). We conclude that the CosΔFu variant does not stabilize Fu in wing discs, consistent with a severe loss of Fu binding.

Surprisingly, Ci-155 protein levels, which are greatly increased in *cos2* mutant clones (Fig. 2D), were restored to normal by expression

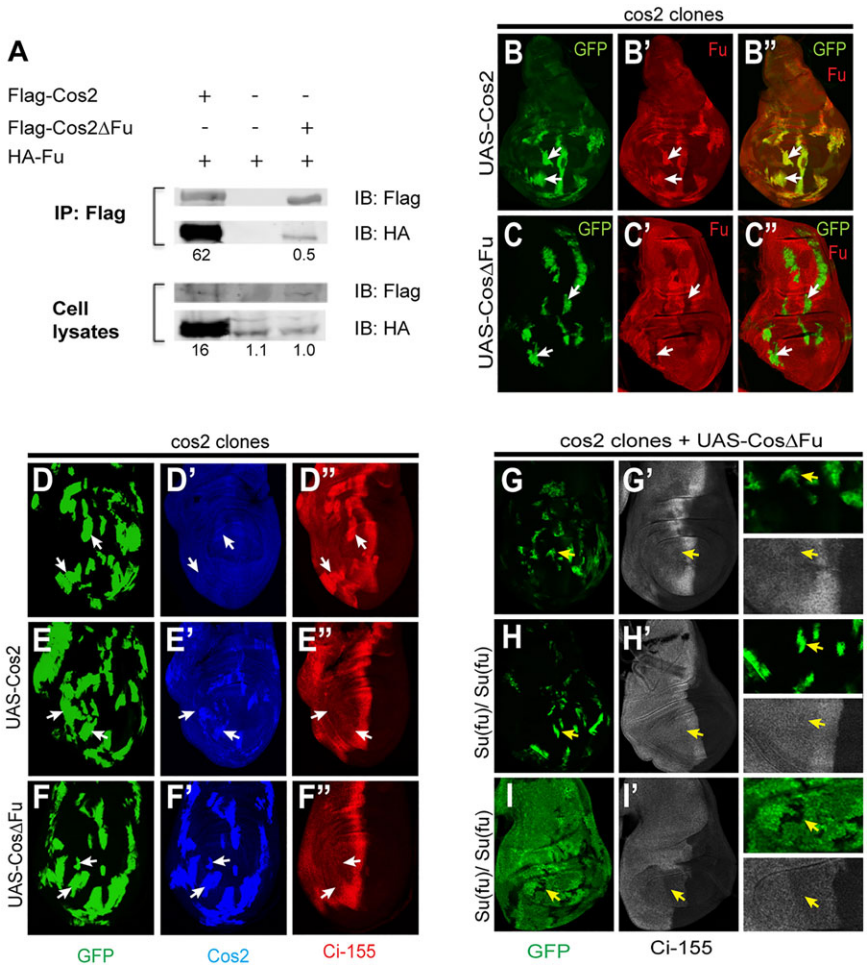


**Fig. 2. Ci-155 processing by overexpressed Cos2 deficient for Fu association.** (A) Kc cells were transfected with DNAs encoding Flag-tagged Cos2 proteins and HA-tagged Fu as shown, followed by immunoprecipitation with Flag antibody (IP: Flag) and western blot with Flag and HA antibodies to measure Fu association with Cos2. Numbers indicate the relative intensity of HA-Fu signals in immunoprecipitates and cell extracts. (B-C″) Fu protein levels (red) were (B-B″) greatly increased in *cos2* mutant clones that express *UAS-cos2* (arrows), marked by GFP (green), but (C-C″) severely reduced in *cos2* mutant clones that express *UAS-CosΔFu*. (D-F) Cos2 levels (blue) were greatly increased in *cos2* clones (arrows), marked by GFP (green), that express (E′) *UAS-Cos2* or (F′) *UAS-CosΔFu* compared with (D′) *cos2* clones alone. Increased Ci-155 (red) in (D″) *cos2* mutant clones was prevented by expression of (E″) *UAS-Cos2* or (F″) *UAS-CosΔFu*. (G-I′) Ci-155 (white) was not elevated in *cos2* mutant clones (arrows), marked by GFP (G-H′) or by the absence of GFP (I,I′), when *UAS-CosΔFu* was expressed in the clone in (G,G′) otherwise normal discs and in (H,H′) *Su(fu)*$^{LP}$ mutant discs, or (I,I′) when *UAS-CosΔFu* was expressed throughout *Su(fu)*$^{LP}$ mutant discs. Insets (right) show clone regions at higher magnification.

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of *UAS-CosΔFu*, just as for wild-type *UAS-Cos2* (Fig. 2E,F). CosΔFu also supported Ci repressor production from both wild-type Ci and Ci-ΔΔ in posterior *smo cos2* mutant clones, yielding strong repression of the *hh-lacZ* reporter (supplementary material Fig. S2A,B,E,F). Thus, when expressed at much higher levels than normal, Cos2 appears to promote efficient Ci-155 processing, despite disruption of its Fu-binding domain.

We then tested whether Fu is recruited to Ci-155 by Su(fu) when CosΔFu replaces wild-type Cos2. First, we found that *hh-lacZ* was still repressed by Ci repressor derived from either wild-type Ci or Ci-ΔΔ in posterior *smo cos2* clones expressing *UAS-CosΔFu* in discs lacking Su(fu) (supplementary material Fig. S2). Second, we saw that Ci-155 levels were still not elevated relative to neighboring tissue in anterior *cos2* clones expressing *UAS-CosΔFu* in discs lacking Su(fu) (Fig. 2G-I). Hence, Su(fu) is not required to support efficient Ci-155 processing even when Cos2, when expressed in excess, lacks an intact Fu-binding site.

### Expression of Cos2 variants at physiological levels to measure *in vivo* function

To test whether efficient Ci-155 processing depended on expressing CosΔFu at abnormally high levels, we sought to express Cos2 variants at physiological levels. A 6.5 kb fragment containing 0.8 kb upstream and 1.7 kb downstream of the *cos2* transcription unit has previously been shown to complement *cos2* function fully (Sisson et al., 1997; Ho et al., 2005). As Cos2 forms dimers (Zhou and Kalderon, 2011; Ranieri et al., 2012) and interacts with many other Hh signaling components, we expected that some variants of Cos2 might dominantly disrupt development, precluding isolation of the desired transgenic flies. We therefore inserted an *FRT*-flanked transcriptional terminator into the first *cos2* intron so that Cos2 expression would be conditional on excision of the *FRT*-flanked cassette. After excising the termination cassette in the female germline we found that none of our Cos2 variants had significant dominant effects. Hence, all experiments were performed with genomic *cos2* transgenes (referred to as *gCos2*) that had undergone germline excision of the transcriptional terminator and retained only an additional *FRT* site and short flanking sequences in the first intron.

The wild-type *gCos2* transgene fully rescued homozygous $cos2^2$ mutant animals to adulthood, producing fertile flies of normal morphology that could be propagated as a stable stock. A single copy of the genomic *CosΔFu* transgene failed to rescue *cos2* homozygotes to adulthood. Wing discs from the latter larvae showed very high levels of Ci-155 throughout the anterior compartment (Fig. 3A,B). Anterior *cos2* mutant clones in *cos2* heterozygotes carrying the genomic *CosΔFu* transgene also had very high Ci-155 levels, in contrast to controls with a wild-type *gCos2* transgene (Fig. 3H-J). The levels of Cos2 protein produced by wild-type *gCos2* and *gCosΔFu* were similar and slightly lower than the levels produced by two copies of the endogenous *cos2* gene (supplementary material Fig. S3D-G). Thus, CosΔFu expressed at physiological levels does not support efficient Ci-155 processing. However, physiological levels of CosΔFu did allow sufficient Ci processing in *smo cos2* clones to repress *hh-lacZ* in animals expressing CiΔΔ in wing discs (supplementary material Fig. S3A,B), mirroring the inefficient Ci-155 processing phenotype of $fu^{M1}$.

In addition to facilitating Ci-155 processing, Cos2 has the potential to limit the activity of Ci-155 as an activator of Hh target genes. Thus, the *ptc* reporter, *ptc-lacZ* is strongly induced in *cos2* mutant clones (Fig. 3L′) because Ci-75 repressor is eliminated, Ci-155 levels are increased and Ci-155 is partially activated (Sisson et al., 1997; Wang and Holmgren, 1999; Wang et al., 2000; Smelkinson et al., 2007). In wing discs with the genomic *CosΔFu* transgene, we did not observe significant *ptc-lacZ* expression in anterior *cos2* mutant clones (Fig. 3N′), suggesting that CosΔFu still inhibits Ci-155 activity even though Ci-155 processing is strongly impaired.

We also constructed and tested a genomic *Cos2-S182N* transgene. The S182N alteration was designed to impair nucleotide binding (Ho et al., 2005) and was later found to prevent Cos2 association with the CORD domain of Ci, which is normally stimulated by nucleotides *in vitro* (Zhou and Kalderon, 2010). Cos2-S182N can promote Ci-155 processing to some degree, but much less efficiently than wild-type Cos2 when overexpressed (Ho et al., 2005; Zhou and Kalderon, 2010). At physiological Cos2-S182N levels, we found that Ci-155 staining was clearly elevated in *cos2* mutant clones (Fig. 3K′) and in the entire anterior compartment of *cos2* homozygotes (Fig. 3C). The genomic *Cos2-S182N* transgene did support strong *hh-lacZ* repression by CiΔΔ in posterior *smo cos2* mutant clones, confirming that it can promote some Ci processing (supplementary material Fig. S3C).

In animals with the *Cos2-S182N* transgene, ectopic *ptc-lacZ* was clearly induced in *cos2* mutant clones (Fig. 3O′), albeit to a level lower than in *cos2* mutant clones with no *cos2* transgene (Fig. 3L′). Thus, Cos2-S182N does not effectively restrict Ci-155 activity. Moreover, direct comparison with CosΔFu, which permits even greater Ci-155 accumulation but no ectopic *ptc-lacZ* induction (Fig. 3J′,N′), shows that the failure of Cos2-S182N to silence Ci-155 in the absence of Hh cannot simply be due to excessive Ci-155 levels or the absence of Ci-75 repressor.

One possible mechanism by which Cos2 restricts Ci-155 activity is by retaining Ci-155 in the cytoplasm. We therefore tested Ci-155 localization in *cos2* clones after incubating discs with the nuclear export inhibitor leptomycin B (LMB). Loss of *cos2* caused increased nuclear accumulation of Ci-155 (Fig. 3D; supplementary material Fig. S3H), as previously reported (Wang and Holmgren, 2000). A similar distribution of Ci-155 was seen in the presence of *gCos-S182N* but Ci-155 was still predominantly cytoplasmic in the presence of *gCosΔFu*, even though overall Ci-155 levels were at least as high (Fig. 3F,G; supplementary material Fig. S3J,K). These data provide the best physiological evidence to date that Cos2 binding to the CORD domain restricts Ci-155 nuclear access and activity.

### Functions of Cos2 in transducing the Hh signal

In response to Hh, Cos2 has been proposed to facilitate Smo activation (Lum et al., 2003), to promote Fu activation (Shi et al., 2011; Zhang et al., 2011; Zhou and Kalderon, 2011) and to alter its activity in response to phosphorylation by Fu (Ruel et al., 2007; Zhou and Kalderon, 2011; Ranieri et al., 2012). However, these hypotheses have not been tested rigorously because overexpression of Cos2, which has been the inevitable consequence of using *UAS-cos2* cDNA transgenes to date, blocks Hh signaling at the AP border of wing discs (Ho et al., 2005; Ruel et al., 2007; Zhou and Kalderon, 2011), while a similar dose effect has been demonstrated in tissue culture (Lum et al., 2003).

We therefore used genomic *Cos2* transgene variants to examine the roles of Cos2 in responding to Hh. In the presence of *gCosΔFu*, *cos2* mutant clones at the AP border showed greatly reduced *ptc-lacZ* staining compared with neighboring AP cells and complete loss of anterior Engrailed (En) expression (Fig. 3L″-N″). Thus, the response to Hh is drastically reduced when Cos2 cannot bind Fu normally. Ci-155 levels were very high and not distinguishably different in anterior and AP border clones expressing only CosΔFu

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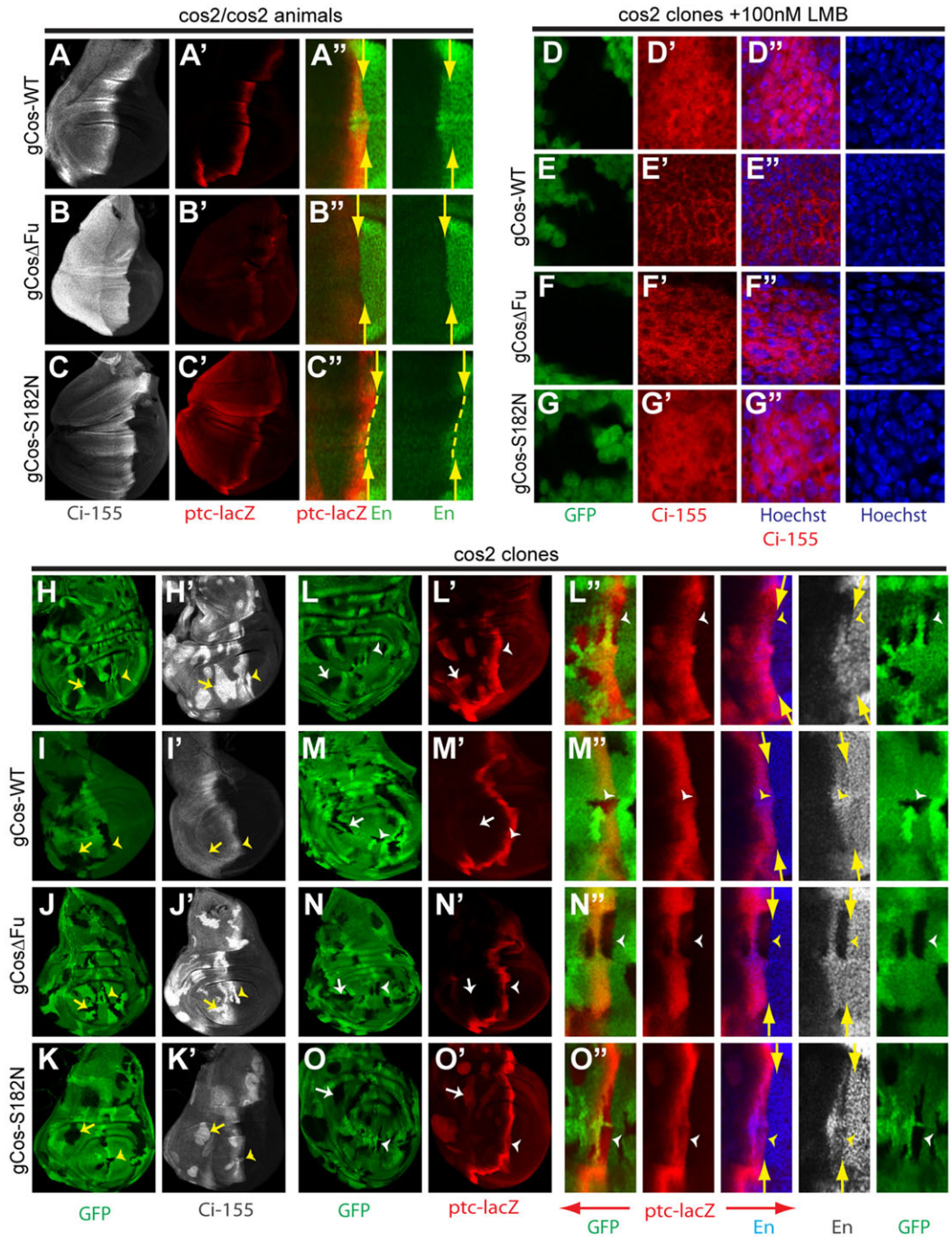


**Fig. 3. Properties of Cos2 variants deficient for Fu or Ci-CORD binding when expressed at physiological levels.** (A-C″) Wing discs homozygous for $cos2^2$ with one copy of genomic transgenes for (A) wild-type Cos2, (B) CosΔFu or (C) Cos2-S182N, showing (A-C) Ci-155 staining (white), (A′-C′) *ptc-lacZ* staining (red) and (A″-C″) En staining (green) alone (right) or together with *ptc-lacZ* (red, left) to reveal the exact position of the AP border (arrows and dashed lines) as the posterior (right) edge of *ptc-lacZ* staining. (D-G″) Wing discs with *cos2* mutant clones marked by loss of GFP (green, D-G) and carrying the indicated transgenes, treated with 100 nM LMB for 2 h. Ci-155 (red, D′-G′) is largely absent from nuclei (blue Hoechst staining, D″-G″) in clones expressing Cos-WT (E′) or CosΔFu (F′) but not Cos2-S182N (G′) or no *gCos2* transgene (D′). (H-O″) Wing discs with *cos2* mutant clones, marked by loss of GFP (green) and (H,L) no Cos2 transgene or one copy of a genomic transgene for (I,M) wild-type Cos2, (J,N) CosΔFu or (K,O) Cos2-S182N, showing (H′-K′) Ci-155 staining (white) and (L′-O′) *ptc-lacZ* staining (red). (L″-O″) Higher magnifications of the AP border in the wing pouch are shown for (from left to right) *ptc-lacZ* (red) and GFP, *ptc-lacZ* alone, *ptc-lacZ* and En (blue), En alone (white), and GFP (green) alone. The AP border is marked with arrows (from *ptc-lacZ* staining) and GFP-negative clones are marked by arrowheads.

(Fig. 3J′). This finding is consistent with strong impairment of Ci-155 processing whether Hh is present or not, and the absence of Cul3-mediated Ci-155 proteolysis normally seen at the AP border when Hh signaling strongly activates Ci-155 (Jiang, 2006). Likewise, in *cos2* homozygous animals with the genomic CosΔFu transgene, Ci-155 levels were uniformly high in anterior and AP border cells, there was no anterior En staining and *ptc-lacZ* expression was confined to a weak AP border stripe, which was wider than normal, presumably because Hh travels further when less Ptc is expressed in AP border cells (Fig. 3A,B).

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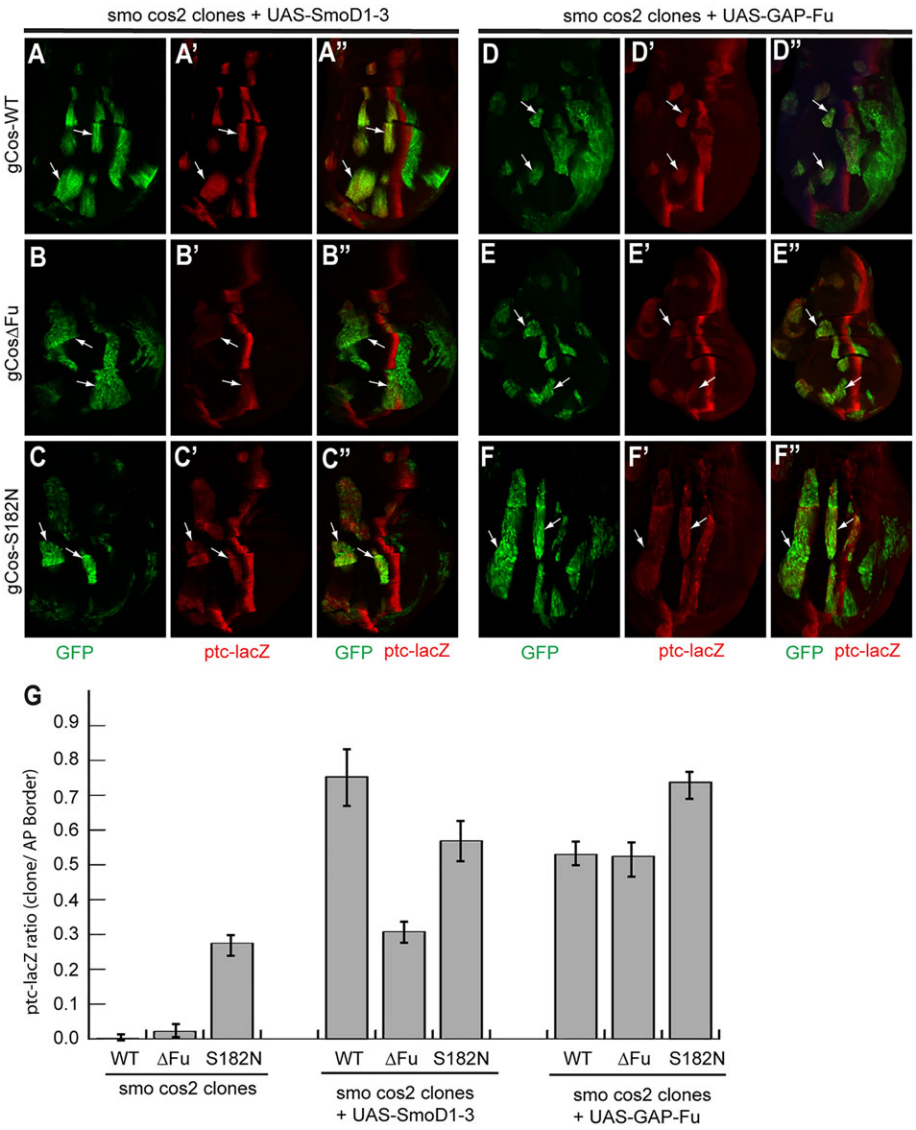


**Fig. 4. Cos2 with impaired Fu binding fails to activate Fu in response to activated Smo.** (A-F″) Ectopic *ptc-lacZ* (red) was induced in anterior GFP-positive (green) *smo cos2* clones by (A-C″) *UAS-SmoD1-3* or (D-F″) *UAS-GAP-Fu* driven by *C765-GAL4* in discs carrying genomic transgenes for (A-A″,D-D″) wild-type Cos2, (B-B″,E-E″) CosΔFu or (C-C″,F-F″) Cos2-S182N. Induction of *ptc-lacZ* by SmoD1-3 was much weaker for (B-B″) CosΔFu than for (A-A″) Cos2-WT or (C-C″) Cos2-S182N, whereas (D-F″) *ptc-lacZ* induction by GAP-Fu was similar for all three Cos2 transgenes. (G) Measurement of *ptc-lacZ* staining in response to SmoD1-3 and GAP-Fu in the presence of the indicated *gCos2* transgenes. Intensity of *ptc-lacZ* staining relative to the AP border calculated from five anterior clones of each genotype is displayed together with the s.e.m. (see Materials and methods).

In wing discs carrying the genomic *Cos2-S182N* transgene, *cos2* clones at the AP border expressed levels of *ptc-lacZ* close to those in normal AP border cells (Fig. 3O′,O″). These clones also expressed anterior En, although often non-uniformly or at lower levels than normal, indicating only very slightly impaired Hh signaling outcomes (Fig. 3O″). A similar, almost complete rescue of anterior En staining by Cos2-S182N was seen in *cos2* homozygous discs (Fig. 3C).

To examine the response of Cos2 variants to Hh in more detail, we examined Smo, responses to activated Smo and responses to activated Fu. When Smo is activated, it accumulates to higher levels, localizes predominantly at the plasma membrane and is more highly phosphorylated (Briscoe and Therond, 2013). Accumulation of activated Smo is readily observed in wild-type wing discs in posterior cells and in the most posterior AP border cells. We found a similar pattern of elevated Smo staining in *cos2* mutant discs carrying wild-type *gCos2* and *gCos-S182N* (supplementary material Fig. S4A,C). Smo staining was also elevated in posterior and AP border cells of *cos2* mutant discs carrying *gCosΔFu*, although elevated Smo staining differed from that of wild-type wing discs by spreading well beyond the range of *ptc-lacZ* induction at the AP border and at ectopic far anterior sites (supplementary material Fig. S4B). Thus, both CosΔFu and Cos-S182N can support at least one aspect of Smo activation, Smo accumulation, in response to Hh.

We then expressed constitutively active Smo (SmoD1-3) in *smo cos2* mutant clones in wing discs carrying each of the three *gCos2* transgenes to test their ability to respond to activated Smo. We found high levels of *ptc-lacZ*, similar to those at the AP border in wild-type cells, in anterior clones expressing SmoD1-3 together with wild-type *gCos2*, slightly lower levels for *gCos2-S182N* and much lower *ptc-lacZ* in the presence of *gCosΔFu* (Fig. 4A-C,G). If the limited response of *ptc-lacZ* to both Hh and activated Smo supported by CosΔFu is only because Fu is not activated in these cells, it should be possible to induce strong *ptc-lacZ* expression in cells with CosΔFu by activating Fu synthetically. The *GAP-Fu* transgene encodes a membrane-tethered Fu fusion protein that induces Fu kinase activation even in the absence of Hh, Smo or Cos2 (Claret et al., 2007; Zhou and Kalderon, 2011). Expression of GAP-Fu in *cos2* clones containing the genomic *CosΔFu* transgene induced strong *ptc-lacZ* expression, just as for *cos2* mutant cells with a wild-type genomic *Cos2* transgene or *gCos2-S182N* (Fig. 4D-G), indicating that the defects of CosΔFu in responding to Hh can be attributed to a failure to activate Fu.

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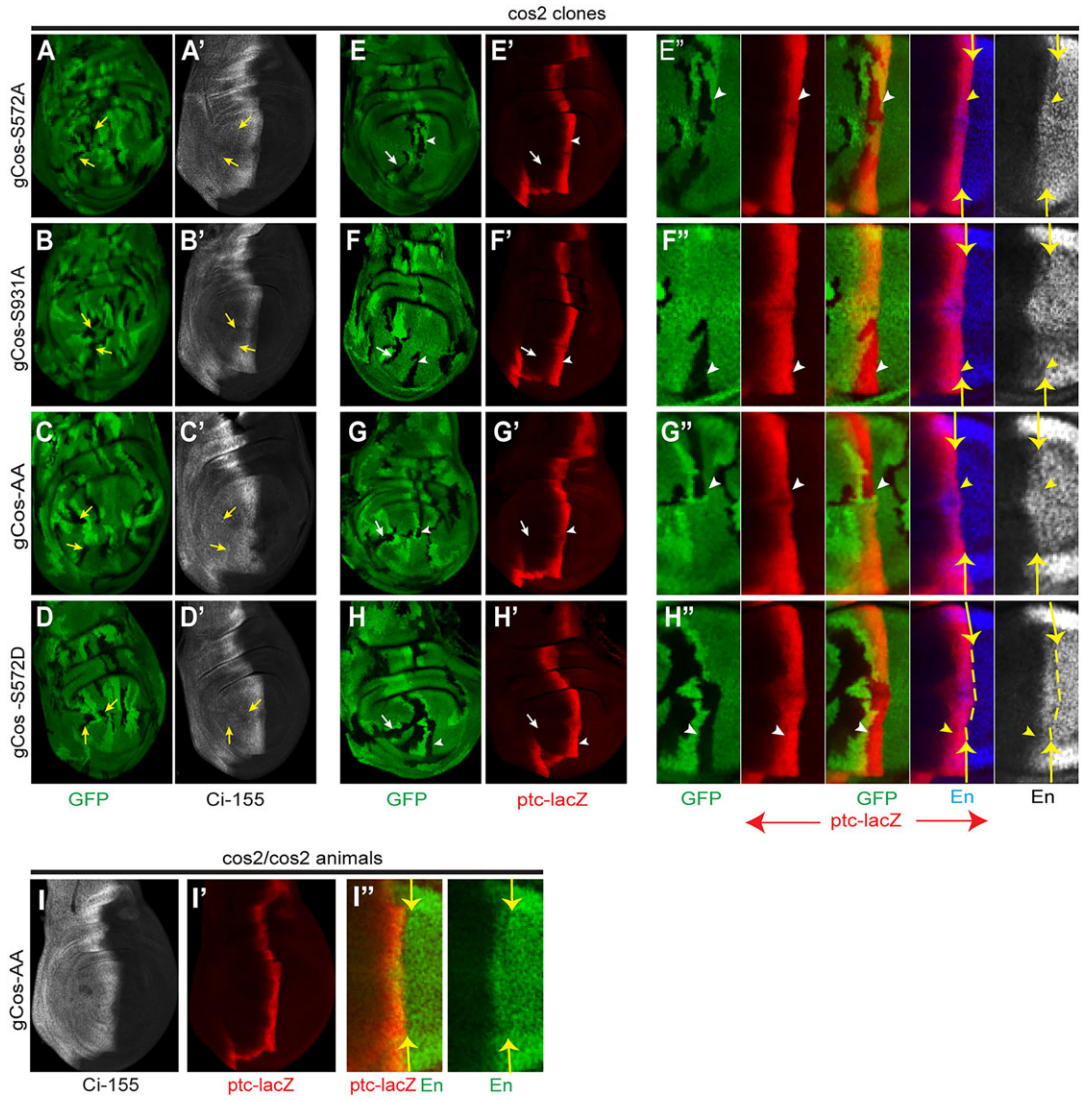


**Fig. 5. Cos2 phosphorylation site variants support normal Hh signaling.** (A-H) Wing discs with *cos2* mutant clones, marked by loss of GFP (green) and one copy of a genomic transgene for (A,E) Cos2-S572A, (B,F) Cos2-S931A, (C,G) Cos2-S572AS931A (Cos2-AA) or (D,H) Cos2-S572D, showing no changes in (A′-D′) Ci-155 (white) or (E′-H′) *ptc-lacZ* (red) staining in anterior (arrows) or AP border clones (arrowheads). (E″-H″) Higher magnifications of the AP border in the wing pouch are shown for (from left to right) GFP (green), *ptc-lacZ* (red), *ptc-lacZ* with GFP, *ptc-lacZ* with En (blue) and En alone (white). The AP border is marked with arrows and dashed lines (from *ptc-lacZ* staining) and GFP-negative clones are marked by arrowheads. (I-I″) Wing disc homozygous for $cos2^2$ with one copy of the g*Cos2-AA* transgene, showing (I) Ci-155 staining (white), (I′) *ptc-lacZ* staining (red) and (I″) En staining (green) alone (right) or (red) together with *ptc-lacZ* (left) to reveal the exact position of the AP border (arrows).

### Physiological function of Cos2 sites phosphorylated by Fu

Two residues on Cos2 have been identified as very likely direct Fu kinase targets (Nybakken et al., 2002). Phospho-specific antibodies demonstrated Cos2 S572 phosphorylation in a wide domain at the AP border of wing discs and S931 phosphorylation in a narrower AP domain where Hh signaling is stronger, with both species detected throughout the posterior compartment (Ruel et al., 2007; Ranieri et al., 2012). Biochemically, it was inferred from co-precipitation measurements and by using Ala or acidic residue substituents that phosphorylation at S572 reduced Cos2 binding to Ci and to Smo (Liu et al., 2007; Ruel et al., 2007). Furthermore, Ci transcriptional activity assays in tissue culture showed that Cos2-S572A did not support a positive response to Fu kinase, whereas wild-type Cos2 did, leading the authors to conclude that S572 phosphorylation was essential for normal activation of Ci-155 by Fu (Ruel et al., 2007). However, these assays used non-physiological levels of Cos2, Fu and Smo. It has also been suggested, based on the properties of overexpressed Cos2 S572 variants in wing discs that phosphorylation of S572 inhibits Ci-155 processing (Ruel et al., 2007; Zhou and Kalderon, 2011).

To test these conclusions in a physiological setting, we used genomic transgenes encoding Cos2-S572A and Cos2-S572D. Surprisingly, we found that both Cos2-S572A and S572D fully suppressed Ci-155 elevation in *cos2* mutant clones, indicating normal Ci-155 processing (Fig. 5A,D). Even more surprising, *cos2* mutant clones at the AP border of discs with genomic *Cos2-S572A* or *S572D* transgenes showed normal strong *ptc-lacZ* expression and anterior En expression (Fig. 5E,H). In fact, *cos2* homozygous animals were fully rescued by each transgene to produce fertile adults of normal morphology. Thus, Cos2 S572 phosphorylation is clearly not essential for Hh to activate target genes appropriately.

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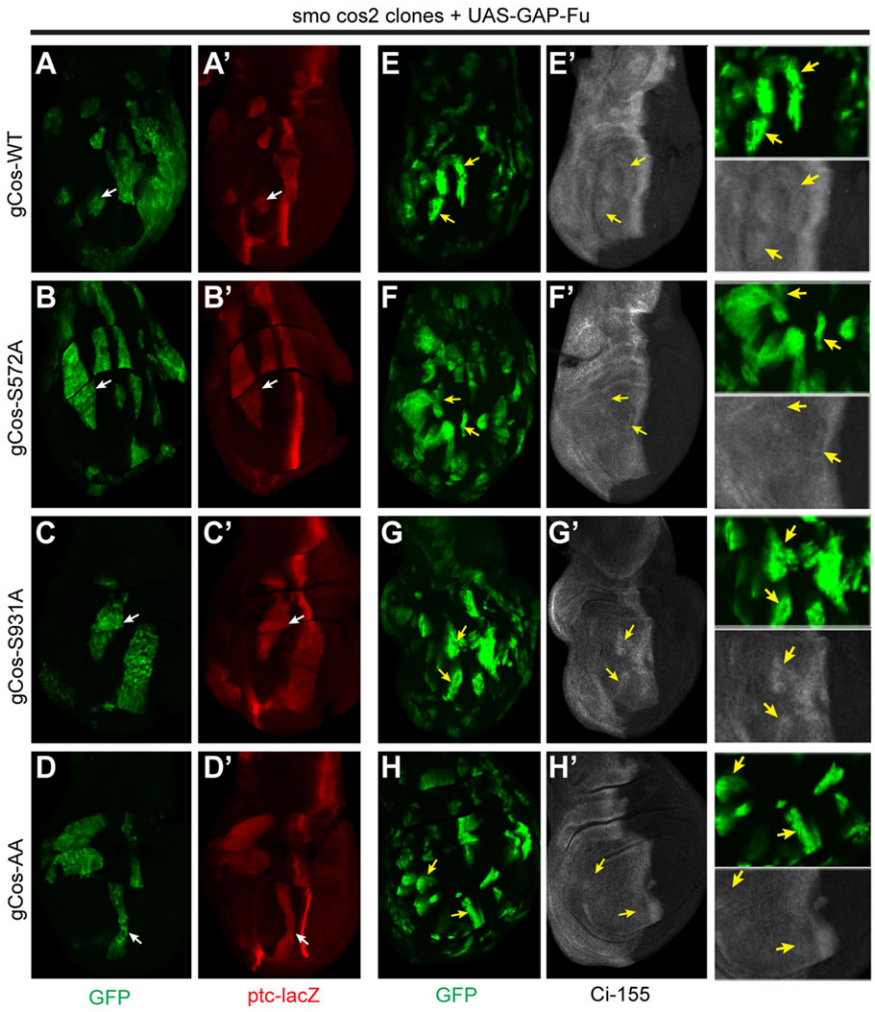


**Fig. 6. Cos2 phosphorylation sites are not required to respond to Fu.** (A-H′) Anterior *smo cos2* clones (arrows) expressing *UAS-GAP-Fu*, marked by GFP (green), in discs carrying genomic transgenes for (A,E) wild-type Cos2, (B,F) Cos2-S572A, (C,G) Cos2-S931A or (D,H) Cos2-AA (S572A S931A) induced (A′-D′) ectopic *ptc-lacZ* (red) to a similar degree. (E′-G′) Ci-155 staining (white) was mildly elevated in most clones of discs with (E′) wild-type *gCos2* and (G′) *gCos2-S931A*, but not with (F′) *gCos-S572A* or (H′) *gCos-AA*. Insets (right) show clone regions at higher magnification.

To test whether Cos2 S572 was important for responses specifically to Fu kinase, we created *smo cos2* mutant clones that expressed GAP-Fu in animals carrying the different genomic *Cos2* transgenes. The *smo* mutation was included to ensure that we measure only downstream responses to Fu activation, as it has been found that active Fu can also contribute to Smo activation (Claret et al., 2007; Liu et al., 2007). Equally strong induction of *ptc-lacZ* was seen for *gCos2-WT* and *gCos2-S572A* transgenes, showing that Ci-155 activation by Fu does not require S572 phosphorylation (Fig. 6A,B). Ci-155 levels were slightly elevated by activated Fu in *cos2* mutant clones with the wild-type *gCos2* transgene but no clear elevation was seen for *gCos2-S572A* (Fig. 6E,F). These results are consistent with S572 phosphorylation being important for Fu kinase to increase Ci-155 levels (Zhou and Kalderon, 2011). Unfortunately the sensitivity of this assay is limited because activated Ci-155 is labile (Ohlmeyer and Kalderon, 1998; Jiang, 2006).

Prior studies of Cos2-S931A and S931D variants in combination with S572A and S572D variants suggested that S931 phosphorylation enhanced Cos2-Smo binding and that it also increased Ci-155 activation, associated with reduced Ci-155 binding and increased Ci-155 nuclear entry (Ranieri et al., 2012). The relevant assays were, however, conducted with non-physiological levels of Cos2, Fu and Smo proteins, principally in tissue culture cells. We found that genomic *Cos2-S931A* and *S931D* transgenes fully rescued *cos2* homozygous mutant animals, producing normal patterns of Hh target gene expression in wing discs (data not shown). Full rescue was also seen for Cos2-S931A when examining *cos2* mutant clones (Fig. 5B,F) and in response to activated Fu in *smo cos2* mutant clones (Fig. 6C,G), as described previously for Cos2-S572A. To determine whether S572 and S931 phosphorylation might act redundantly, we also tested Cos2-AA (S572A S931A). This genomic transgene was constructed directly without a transcriptional terminator cassette, along with an analogous wild-type *cos2* transgene. Both transgenes rescued *cos2* mutant clones (Fig. 5C,G) and *cos2* homozygous mutant animals fully (Fig. 5I), producing normal adult wing morphologies and normal patterns of Hh target gene expression in wing discs. Furthermore, Cos2-AA supported strong *ptc-lacZ* induction by activated GAP-Fu in *smo cos2* mutant clones with no significant increase in Ci-155 levels, just like Cos2-S572A (Fig. 6D,H). We also tested the response to a different activated Fu kinase transgene, *Fu-EE*, with similar results. Fu-EE, like GAP-Fu, produced a small increase in Ci-155 levels in the presence of the wild-type Cos2 transgene but no clear increase in the presence of Cos2-AA (supplementary material Fig. S5A,B), providing further evidence that Cos2 phosphorylation is required for Fu kinase to increase Ci-155 levels.

### Su(fu) and Cos2 do not appear to be redundant targets for responses to Fu kinase activity

As Su(fu), as well as Cos2, becomes hyper-phosphorylated during Hh signaling in a Fu-dependent manner (Lum et al., 2003; Ho et al., 2005), it was thought that Su(fu) phosphorylation probably contributes to Hh signal transduction, specifically in response to

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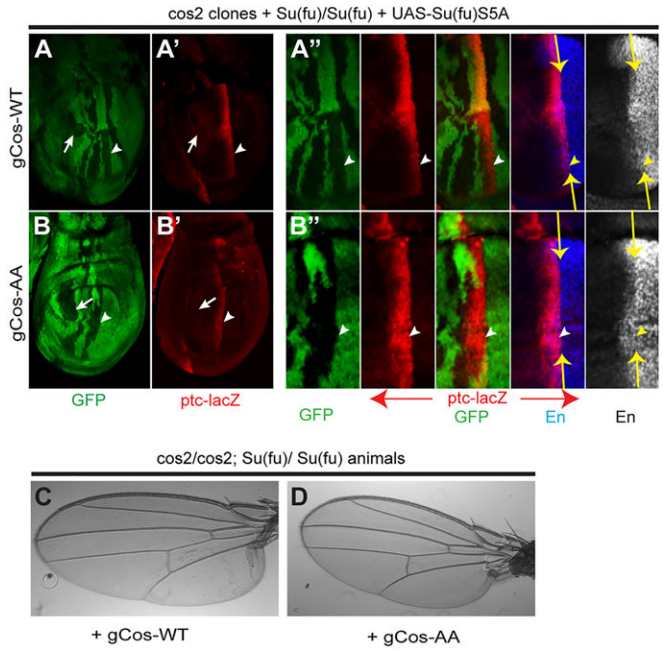


**Fig. 7. Cos2 phosphorylation sites are not required to respond to Hh even in the absence of normal Su(fu) phosphorylation.** (A,B) *Su(fu)* mutant wing discs with *UAS-Su(fu)-5A* (which lacks known Fu phosphorylation sites) expressed ubiquitously using *C765-GAL4*, and one copy of a genomic transgene for (A) wild-type Cos2 or (B) Cos2-AA. In *cos2* mutant clones, marked by loss of GFP (green) (A′,B′), *ptc-lacZ* staining (red) was unchanged. (A″,B″) Higher magnifications of the AP border in the wing pouch are shown for (from left to right) GFP (green), *ptc-lacZ* (red), *ptc-lacZ* with GFP, *ptc-lacZ* with En (blue) and En alone (white). The AP border is marked with arrows (from *ptc-lacZ* staining) and GFP-negative clones are marked by arrowheads. (C,D) Wings from $cos2^{2}/cos2^{2}$; $Su(fu)^{LP}/Su(fu)^{LP}$ animals carrying two copies of the (C) *gCos-WT* or (D) *gCos-AA* transgene on the second chromosome.

activated Fu. However, after identifying the major Fu-dependent phosphorylation sites on Su(fu), it was found that altering those sites had no effect on Hh signaling or responses to Fu (Zhou and Kalderon, 2011). We therefore removed the known Fu phosphorylation sites on Su(fu) and on Cos2 simultaneously in case these two targets of Fu act redundantly. We found that *cos2* clones in the anterior and at the AP border were still fully rescued by the genomic *Cos2-AA* transgene in wing discs homozygous for the null $Su(fu)^{LP}$ mutation but expressing a Su(fu) variant (Su-5A) with five serine residues (constituting Fu phosphorylation sites and neighboring residues) altered to alanine (Zhou and Kalderon, 2011) (Fig. 7A,B). Furthermore, animals containing Cos2-AA and Su-5A as the only functional versions of Cos2 and Su(fu) developed into adults of normal morphology. Similar results were seen in Su(fu)-null animals expressing only Cos2-AA (Fig. 7C,D). Thus, even though Fu kinase activity is essential for normal responses to Hh, neither the known Fu phosphorylation sites on Cos2 nor those on Su(fu) are required to respond to Hh.

## DISCUSSION

We have investigated the role of Cos2 in Hh signaling for the first time using variants expressed at normal levels in *Drosophila* tissues. This approach is crucial because Cos2 collaborates with several direct binding partners and its actions have been found to be extremely dose sensitive. Our results provide definitive evidence for prior proposals that Cos2 association with Fu is essential for Ci-155 processing (in the absence of Hh) and for Fu kinase activation (in response to Hh), and suggest that Cos2 binding to the CORD domain of Ci-155 promotes both Ci-155 processing and Ci-155 silencing. Our results also contradict prior assertions that phosphorylation of specific Cos2 sites (S572 and S931) by Fu is essential for Ci-155 activation in response to Hh.

### Functions of Cos2, Fu and Su(fu) in Ci-155 processing and silencing in the absence of Hh

Cos2 is generally regarded as the major mediator of Ci-155 processing (Zhang et al., 2005), whereas the role of Fu is less clear. Here, we find that three class II *fu* alleles (encoding truncated proteins) strongly impair Ci-155 processing in a cell-autonomous manner, whereas even $fu^{M1}$, which encodes only 80 amino acids of the N-terminal kinase domain, still permits a low level of Ci-155 processing. All of these *fu* alleles encode proteins lacking a C-terminal Cos2-binding domain (Robbins et al., 1997; Monnier et al., 2002), suggesting that a low level of Ci-155 processing can occur in the absence of Fu but efficient processing requires Fu to bind to Cos2. Our evidence specifically argues against previous assertions that Fu is absolutely required for Ci-155 processing (Methot and Basler, 2000) and that some Fu proteins that cannot bind Cos2 are nevertheless able to promote efficient Ci-155 processing in the absence of Hh (Wang and Holmgren, 1999). The requirement for Fu in Ci-155 processing may be explained by the recent finding that Fu, rather than Cos2, is principally responsible for recruiting PKA to Ci-155 (Ranieri et al., 2014).

Consistent with our deductions from *fu* alleles, we found that a Cos2 variant with strongly impaired Fu binding (CosΔFu) supported low-level Ci-155 processing (detected by a sensitive *hh-lacZ* repression assay) but not efficient Ci-155 processing (measured by Ci-155 staining) when CosΔFu was expressed at physiological levels. Surprisingly, Ci-155 processing appeared normal when CosΔFu was strongly overexpressed. Given the failure of excess Cos2 to rescue the processing defects of $fu^{M1}$, we suggest that some Fu protein is recruited by CosΔFu to Ci-155 processing complexes when CosΔFu is overexpressed, likely due to some residual direct binding between Fu and CosΔFu. Ci-155 processing by excess CosΔFu was not detectably impaired by eliminating Su(fu), suggesting that Su(fu) does not contribute to Fu recruitment even under conditions of greatly reduced Cos2-Fu binding, despite the potential for Su(fu) to bind to both Fu and Ci-155. It appears therefore that Su(fu) has no detectable role in Ci-155 processing, in contrast to evidence of an important role in Gli2/3 processing in mammals (Kise et al., 2009; Humke et al., 2010).

Three domains on Ci-155 that bind Cos2 have been identified biochemically but Ci-155 processing was disrupted in wing discs only when two of these (the zinc fingers and the CORD domain) were deleted simultaneously, with no discernible negative consequence of deleting the third (CDN) region (Wang et al., 2000; Wang and Jiang, 2004; Zhou and Kalderon, 2010). Surprisingly, deletion of the CORD domain, especially in combination with the CDN region, appears to enhance Ci-155 processing (Zhou and Kalderon, 2010). It was therefore suggested that Cos2 binding to CORD might compete with alternative CORD binding interactions, which might otherwise reduce processing, perhaps by masking PKA, CK1 or GSK3 phosphorylation sites. A key test of that idea involved Cos2-S182N, which cannot bind the CORD region but still associates normally with other regions of Ci-155 (Zhou and Kalderon, 2010). Here, we found that Cos2-S182N expressed at physiological levels supported only low levels of Ci-155 processing. Moreover, excess Ci-155 spared from processing induced low levels of *ptc-lacZ*, whereas a similar processing defect due to CosΔFu resulted in no ectopic *ptc-lacZ*. CosΔFu also retained Ci-155

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in the cytoplasm more effectively than Cos2-S182N in wing discs treated with the nuclear export inhibitor LMB. Hence, it appears that Cos2 binding to CORD normally promotes Ci-155 processing and also limits the nuclear access and transcriptional activity of Ci-155 when Ci processing is impaired. This suggests that Cos2-CORD interaction may be regulated during Hh signaling in order for Ci-155 to be fully activated.

### Functions of Cos2-Fu association

Recently, the mechanism of Fu kinase activation has come into focus. Fu kinase activation depends on phosphorylation of putative Fu and CK1 sites in the activation loop of Fu (Shi et al., 2011; Zhang et al., 2011; Zhou and Kalderon, 2011). It is promoted by association of Fu molecules and requires the region of Fu that binds to Cos2, suggesting that activated Smo clusters Cos2-bound Fu molecules to stimulate their cross-phosphorylation and consequent full activation. Here, we provide further evidence for that model by showing that Cos2 deficient for binding to Fu (CosΔFu) fails to support strong Ci-155 activation in response to Hh or activated Smo.

We found that Cos2 must also bind to Fu to stabilize Fu. What might be the purpose of Fu stabilization by Cos2? One possible purpose is to prevent spurious Fu activation. Fu is activated by aggregation and a positive-feedback loop of cross-phosphorylation. By ensuring that excess Fu is unstable and almost all Fu molecules are associated with Cos2, Fu activation can be limited to circumstances where Cos2 molecules are aggregated. Conversely, stabilization of Fu by Cos2 allows the relatively low abundance Cos2 protein to compete better with other potential Fu partners for Fu binding, thereby priming Fu for Cos2-dependent activation. A second potential purpose of Fu stabilization by Cos2 concerns Ci-155 processing. If Cos2 and Fu each recruit kinases to Ci-155 and an effective processing complex must contain multiple components (Ci-155, Cos2, Fu, PKA, CK1 and GSK3) stabilization of Fu by binding to Cos2 would ensure that productive associations of Fu with PKA, CK1 or GSK3 are not distributed throughout the cell but concentrated in Ci-Cos2-Fu complexes. It also seems likely, though currently untested, that the presence of Fu enhances Cos2 association with Ci-155. Otherwise it would be hard to understand why excess Cos2 does not impair Ci-155 processing by forming a high proportion of Ci-155 complexes lacking Fu, or how excess CosΔFu can process Ci-155 efficiently despite extremely low levels of Fu.

### Mechanism of Ci-155 activation by Fu kinase

As both Su(fu) and Cos2 bind directly to Ci-155 and both have been shown to limit nuclear accumulation and activity of Ci-155 under some conditions, it has been suggested that Fu kinase might overcome these inhibitory actions by phosphorylating one or more of these components directly. Su(fu) is phosphorylated in response to Hh and activated Fu (Lum et al., 2003; Ho et al., 2005), but alteration of the identified phosphorylation sites had no measurable effect on either the silencing activity of Su(fu) or on the ability of Hh or Fu to oppose that silencing (Zhou and Kalderon, 2011). By contrast, the two identified sites of Hh-stimulated Fu-dependent phosphorylation on Cos2 appeared to play a significant role (Ruel et al., 2007; Ranieri et al., 2012). It was variously argued that Cos2 S572 phosphorylation contributed to Smo activation, promoted Cos2 degradation, reduced association with Ci-155 and blocked Ci-155 processing (Liu et al., 2007; Ruel et al., 2007; Zhou and Kalderon, 2011). Here, we found that an S572A substitution in Cos2 did not impair Ci-155 processing or silencing in the absence of Hh or any aspect of the response to Hh, allowing the development of morphologically normal and fertile adults.

We did find some support for the assertion that Ci-155 stabilization by activated Fu depends on S572 phosphorylation (Zhou and Kalderon, 2011), seemingly consistent with the finding that Cos2-S572D associates poorly with Ci-155 in co-precipitation experiments (Ruel et al., 2007). However, here we found that physiological levels of Cos2-S572D supported efficient Ci-155 processing. In addition, the impaired Ci association of Cos2-S572D seen by co-precipitation of tissue culture cell extracts (Ruel et al., 2007) was not observed using *in vitro* binding experiments that employed isolated Cos2-binding domains of Ci (Zhou and Kalderon, 2010). Hence, it remains unclear whether the S572D substitution is a good mimic of phosphorylation or exactly how S572 phosphorylation might affect Cos2 binding to Ci-155 or to other partners in order to impair Ci-155 processing.

Phosphorylation of Cos2 on S931 was suggested to be important for high-level Hh signaling (Ranieri et al., 2012). This idea was supported by transcriptional reporter assays in tissue culture cells that used transfected Cos2 variants together with carefully titrated doses of Smo and Fu. Increased transcriptional response for phosphomimetic alterations and impaired responses for Ala substituents in these experiments were ascribed principally to alterations of S931, although the most clear-cut differences were seen only when both S572 and S931 were altered in analogous fashion (Ranieri et al., 2012). We found that both Cos2-S931A and Cos2-AA (S572A S931A) behaved exactly like wild-type Cos2 in silencing Ci-155 in the absence of Hh, supporting a normal Hh response at the AP border of wing discs and allowing the development of morphologically normal fertile adult flies. The only minor deficit noted was a failure of Cos2-AA to support a small increase in Ci-155 levels in response to activated Fu, exactly as seen for Cos2-S572A.

There is some evidence that the response to Hh at the AP border of wing discs may include some redundancy. For example, it appears that Fu kinase activity can inhibit Ci-155 processing (via Cos2 S572 phosphorylation) but Fu kinase is not necessary for Hh to inhibit Ci-155 processing fully (Zhou and Kalderon, 2011). We therefore tested whether altering Fu phosphorylation sites on both Su(fu) and Cos2 might reveal some redundant actions. However, we saw no abnormalities in Hh responses in *cos2* mutant clones at the AP border of wing discs expressing Cos2-AA and Su(fu) with Ala substituents at phosphorylation sites in place of endogenous Su(fu). Conceivably, a third set of Fu-dependent changes may need to be blocked in order to reveal a contribution of the known Fu phosphorylation sites on Cos2 or Su(fu). For the present, it is clear that key targets of Fu kinase remain to be identified.

## MATERIALS AND METHODS

### Mutagenesis and cloning

A 6.5 kb fragment of genomic Cos2, extending between two *Kpn*I sites in the plasmid vector pCaSpeR4 had been used to rescue Cos2 function in flies (Sisson et al., 1997). We introduced modifications in order to incorporate this fragment into an ATT vector, add an excisable transcription termination cassette in the first intron, and modify Cos2-coding sequence, all by using oligonucleotide-mediated mutagenesis (QuikChange, Stratagene), as detailed in the methods in the supplementary material.

### Cell culture, transfection, immunoprecipitation and western blot analysis

Kc cells were kept at 25°C in Schneider's Drosophila media +5% FBS +1% penicillin-streptomycin (Gibco). Three 10 cm plates were seeded with $1\times10^7$ cells and were given fresh media after 24 h. The cells were transfected

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3-4 h later with *Actin-HA-Fu* together with *Actin-Flag-Cos2*, *Actin-HA-Fu* or *Actin-Flag- CosΔFu* (8 µg each) using a calcium phosphate protocol (Invitrogen). Cells were given fresh medium 24 h later and were harvested after 48 more hours. The cells were lysed at 4°C in 1 ml lysis buffer [50 mM Hepes (pH 7.5), 1.25 mM EDTA, 50 mM NaF, 0.5 mM $Na_2VO_3$ 0.5% NP-40, 1 mM DTT, protease inhibitors (mini complete, Roche)]. The lysates were incubated with mouse anti-Flag antibody conjugated to agarose beads (Sigma) for 2 h at 4°C. The immunoprecipitates were washed three times for 10 min in lysis buffer. The western blots were probed with mouse anti-Flag and rabbit anti-HA antibody (ab9110, Abcam). Secondary antibodies Alexa Fluor-680 and Alexa Fluor-800 were visualized with LI-COR Infrared imager, and the bands were quantified using LI-COR Odyssey Software.

### Immunohistochemistry

Wing disc clones were generated by heat-shocking late first or early second instar larvae for 1-2 h at 37°C. Late 3rd instar larvae were dissected into 4% paraformaldehyde on ice and rocked at 25°C for 30 min. For measurements of Ci-155 nuclear access, discs were dissected in cold PBS and treated with 100 nM LMB (or no LMB as a control) in Schneider's Drosophila cell media for 2 h prior to fixation. Fixed samples were rinsed with 1× PBST (1× PBS +0.1% Triton X-100+0.05% Tween 20) and placed in the blocking buffer [5% goat serum, 0.5% BSA, 50 mM Tris (pH 7.0), 150 mM NaCl, 0.5% BSA] for 2 h at 4°C. The discs were stained with rabbit (MP Biomedicals) and mouse (Promega) anti-β-galactosidase antibody for *lacZ* products, mouse monoclonal 4A6 antibody to Myc (Millipore), rat monoclonal 2A1 antibody to Ci, mouse monoclonal antibodies 17E11 to Cos2 and 4D9 to En (Developmental Studies Hybridoma Bank, University of Iowa, USA), mouse monoclonal antibody 5D6 to Cos2, and rabbit antibody to Fu (Ascano et al., 2002). Secondary antibodies were Alexa Fluor-488, -546, -594, -647 or -680 (Molecular Probes). A Zeiss LSM700 confocal microscope was used to examine the fluorescent staining.

### Measurements from fluorescent images

In Fig. 1, the areas within a rectangle were quantified and intensity plots for Ci-155 staining were constructed using Image J software (NIH, Bethesda, MD). The *y*-axis shows the average fluorescence intensity over the height of the rectangle at each point on the *x*-axis.

In Fig. 4, the average fluorescent intensity (I) of a fixed area within GFP-expressing clones and the normal AP border (no clones) was measured using Image J software. Five separate clones (from at least three wing discs) were measured for each genotype, together with five samples at the AP border and of anterior territory with no clone ('background'). The signal due to each clone was calculated relative to the signal due to Hh at the AP border as $(I_{clone}-I_{background})/(I_{AP\ Border}-I_{Background})$ before deriving the mean signal and standard error for each clone genotype.

### Fly crosses

Transgenic flies and genotypes for all experiments are described in the methods in the supplementary material.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

E.V.Z. and D.K. initially planned the strategy, E.V.Z. and J.L. performed the experiments, and E.V.Z., J.L. and D.K. contributed to the manuscript.

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#### Supplementary material

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# Chapter 3

This was a collaborative effort between Elisa, Dan, and me. I began this project with Qianhe Zhou by performing the genetic screen. Elisa did most of the cloning of gCi transgenes. I primarily worked on the figures involving Srp54 while Elisa primarily worked on the figures with Mago and the EJC. In the final stages of the paper, Elisa put together the Figures and I performed the last sets of experiments. Elisa, Dan, and I maintained the fly stocks and set up the experiments.

# The Exon Junction Complex and Srp54 Contribute to Hedgehog Signaling via *ci* RNA Splicing in *Drosophila melanogaster*

**Elisa Garcia-Garcia,[1] Jamie C. Little,[1] and Daniel Kalderon[2]**
Department of Biological Sciences, Columbia University, New York, 10027
ORCID IDs: 0000-0002-3587-0474 (E.G.-G.); 0000-0002-2149-0673 (D.K.)

**ABSTRACT** Hedgehog (Hh) regulates the Cubitus interruptus (Ci) transcription factor in *Drosophila melanogaster* by activating full-length Ci-155 and blocking processing to the Ci-75 repressor. However, the interplay between the regulation of Ci-155 levels and activity, as well as processing-independent mechanisms that affect Ci-155 levels, have not been explored extensively. Here, we identified Mago Nashi (Mago) and Y14 core Exon Junction Complex (EJC) proteins, as well as the Srp54 splicing factor, as modifiers of Hh pathway activity under sensitized conditions. Mago inhibition reduced Hh pathway activity by altering the splicing pattern of *ci* to reduce Ci-155 levels. Srp54 inhibition also affected pathway activity by reducing *ci* RNA levels but additionally altered Ci-155 levels and activity independently of *ci* splicing. Further tests using *ci* transgenes and *ci* mutations confirmed evidence from studying the effects of Mago and Srp54 that relatively small changes in the level of Ci-155 primary translation product alter Hh pathway activity under a variety of sensitized conditions. We additionally used *ci* transgenes lacking intron sequences or the presumed translation initiation codon for an alternatively spliced *ci* RNA to provide further evidence that Mago acts principally by modulating the levels of the major *ci* RNA encoding Ci-155, and to show that *ci* introns are necessary to support the production of sufficient Ci-155 for robust Hh signaling and may also be important mediators of regulatory inputs.

**KEYWORDS** Hedgehog signaling; exon junction complex; cubitus interruptus; splicing; *Drosophila*

HEDGEHOG (Hh) signaling plays many roles in development and tissue maintenance from *Drosophila* to humans. Accordingly, genetic mutations that alter Hh signaling are associated with a wide range of birth defects and cancers, some of which are being treated with drugs that inhibit Hh signaling (Anderson *et al.* 2012; Petrova and Joyner 2014; Pak and Segal 2016). In *Drosophila* and in mammals, cells respond to Hh primarily by altering the activity of Gli-family transcription factors (Hui and Angers 2011; Xiong *et al.* 2015; Pak and Segal 2016). In the absence of Hh, the primary translation products of *Drosophila* Cubitus interruptus (Ci), as well as mammalian Gli-2 and Gli-3 orthologs, are proteolytically processed to C-terminally truncated forms that readily enter the nucleus and repress Hh target genes, while unprocessed full-length proteins remain largely cytoplasmic and inactive. When Hh binds to Patched (Ptc) and its coreceptors, the seven-transmembrane domain Smoothened (Smo) protein is activated with two key consequences; inhibition of Ci/Gli-2/3 processing and activation of full-length Ci/Gli transcriptional activators, including Gli-1, which is not subject to processing.

Ci/Gli processing involves phosphorylation by Protein Kinase A (PKA) and other protein kinases, scaffolded by a kinesin-like protein (Cos2/Kif7), to create a Cul1-dependent E3 ubiquitin ligase binding site; Hh is thought to inhibit Ci-155 processing by promoting dissociation of these phosphorylation complexes (Hui and Angers 2011; Xiong *et al.* 2015; Pak and Segal 2016). Inhibition of Ci-155 processing reduces or eliminates Ci-75 repressor but also increases Ci-155 levels. Ci-75 repressor maintains some key Hh target genes silent outside Hh signaling territory, but the relative importance of eliminating the repressor and increasing the levels of Ci-155 in cells responding to Hh has not been satisfactorily determined. High levels of Hh also promote Ci-155 degradation via a Cul3-dependent E3 ubiquitin ligase that has been


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[1]These authors contributed equally to this work.
[2]Corresponding author: Department of Biological Sciences, Columbia University, 1013 Fairchild Bldg., 1212 Amsterdam Ave., New York, NY 10027. E-mail: ddk1@columbia.edu

considered as a possible negative feedback mechanism for limiting Ci-155 activity (Ohlmeyer and Kalderon 1998; Kent *et al.* 2006; Zhang *et al.* 2006). Altogether, the contribution of changes in Ci-155 levels to Hh signaling are complex and have been hard to assess.

The mechanism of full-length Ci/Gli activation is understood only in outline. It is thought principally to involve relief of inhibition by Suppressor of fused [Su(fu)], which binds directly to Ci/Gli proteins and, in *Drosophila*, it depends on Hh-activated Fused (Fu) protein kinase activity (Humke *et al.* 2010; Tukachinsky *et al.* 2010; Zhou and Kalderon 2011; Han *et al.* 2015; Oh *et al.* 2015; Zhang *et al.* 2016). It is also unclear to what degree regulation of Ci-155 activation, Ci-155, and Ci-75 levels must collaborate to produce graded Hh signaling. Dose-dependent signaling can be studied in wing discs by the quantitative induction of the universal Hh target gene, *ptc*, and the induction of target genes induced by low levels (*decapentaplegic*; *dpp*), intermediate levels (*collier*; *col*), or only by high levels (*engrailed*; *en*) of signaling (Vervoort 2000). There is some evidence of apparent redundancy, perhaps as a means to support robust signaling in a variety of settings. For example, Hh signaling is normal in the absence of Su(fu), suggesting that regulation of Ci-155 activation can be largely dispensable (Preat 1992; Ohlmeyer and Kalderon 1998). Conversely, synthetic activation of Fu kinase, which promotes Ci-155 activity, can suffice to induce strong Hh pathway activity without the strong inhibition of Ci-155 processing that normally accompanies Hh signaling (Zhou and Kalderon 2011).

To uncover new insights into the regulation of Ci-155 levels and activity we conducted a genetic screen in a sensitized background of diminished Hh signaling lacking any regulatory input from Fu kinase activity. Surprisingly, the screen revealed proteins involved in RNA processing, including core components of the Exon Junction Complex (EJC), which has recently been implicated in regulating RNA splicing (Roignant and Treisman 2010; Ashton-Beaucage and Therrien 2011; Hayashi *et al.* 2014; Malone *et al.* 2014; Le Hir *et al.* 2016), and the serine-arginine rich (SR) protein, Srp54. We provide evidence that the EJC and Srp54 target *ci* splicing to influence Hh signaling by altering the levels of Ci-155 primary translation product. We also explore the role of an alternative *ci* RNA and its presumed translation product.

## Materials and Methods

### *Drosophila stocks*

*Drosophila* stocks were maintained on standard cornmeal/molasses/agar medium at room temperature. For the modifier screen, *yw hs-flp* $fu^{mH63}$; *FRT42D P*[$Fu^{+}$, $w^{+}$] *P*[$y^{+}$]/*Cyo*; *Su*(*fu*)$^{LP}$ *C765-GAL4 ptc-lacZ/TM6B* females were crossed to males with second or third chromosome deficiencies over balancer chromosomes from the Bloomington deletion library (Ryder *et al.* 2007) to produce male progeny lacking Fu kinase activity and heterozygous for both *Su*(*fu*) and the tested deficiency. The same females were crossed to *UAS-RNAi* (*upstream activating sequence-RNA interference*) stocks with or without *gCi* transgenes (16-kb genomic segments constructed as described in the section below) or $ci^{94}$/*Dp*(*1*;*4*)*1021*, $y^{+}$, $sv^{spa\text{-}pol}$ to examine wing discs from male *y* (*fu* mutant) larval progeny. To analyze adult *fu* mutant male progeny with straight wings, the *CyO* balancer was replaced with *Sp* in the parental females.

*yw hs-flp*; *Sp/Cyo*; *C765-GAL4 ptc-lacZ/TM6B* females were crossed to *UAS-RNAi* transgenes (with or without *gCi* transgenes or *UAS-Srp54* or *UAS-CG3605* transgenes) to examine wing discs of progeny with normal Fu and Su(fu) activities. *C765-GAL4* is expressed throughout developing wing discs. *UAS-RNAi* lines for *CG3605* (GD-26250) and *Srp54* (GD-51088) were recombined with *UAS-Diap1* on the third chromosome (BL-6657); second chromosome *UAS-mago RNAi* and *UAS-Y14 RNAi* were provided by J. E. Treisman (Roignant and Treisman 2010) and other RNAi lines tested were from the Bloomington *Drosophila* Stock Center or from the Vienna *Drosophila* Resource Center (prefaced by GD or KK) (Dietzl *et al.* 2007), including those for *eIF4AIII* (KK-108580) and *btz* (GD-38722).

Clones in a Minute background were made by crossing *yw hs-flp*; *FRT42D M*(*2*)*53*[*1*] *P*[*hs-GFP*, $y^{+}$]; *ptc-lacZ/TM6B* females to *FRT42D* $Y14^{\Delta 18}$ *dark/CyO* males from J. E. Treisman (who also provided *UAS-MAPK* stocks) (Roignant and Treisman 2010).

To generate MARCM (mosaic analysis with a repressible clone marker) clones with activated Fu (and loss of *smo* activity) with or without *UAS-RNAi* expression (*UAS-Diap1* was used as a control whenever testing *UAS-Srp54 RNAi* plus *UAS-Diap1*), *yw hs-flp UAS-GFP*; $smo^{2}$ *FRT42D P*[$smo^{+}$] *tub-Gal80*; *C765 ptc-lacZ/TM6B* females were crossed to *yw*; $smo^{2}$ *FRT42D* (*UAS-mago RNAi*) *UAS-GAP-Fu*; (*UAS-Diap1*) (*UAS-Srp54 RNAi*) males. For *smo cos2* clones and *ptc* clones, males had $cos2^{2}$ or $ptc^{S2}$, respectively, in place of *UAS-GAP-Fu*.

For *pka* clones, *yw hs-flp UAS-GFP*; *tub-Gal80 FRT40A*; *C765 ptc-lacZ/TM6B* females were crossed to *yw hs-flp*; *pka-*$C1^{H2}$ *FRT40A* (*UAS-mago RNAi*)*/Cyo*; (*UAS-Diap1*) (*UAS-Srp54 RNAi*) males. In all cases, clones were induced by a 1-hr heat-shock at 37° of first and second instar larvae.

To test replacement of *ci* with *gCi* or *UAS-Ci* transgenes in MARCM clones, *yw hs-flp UAS-GFP*; *FRT42D P*[$ci^{+}$, $w^{+}$] *tub-GAL80/CyO*; *C765-GAL4 ptc-lacZ/TM6B*; $ci^{94}$/*Dp*(*1*;*4*)*1021*, $y^{+}$, $sv^{spa\text{-}pol}$ females were crossed to *yw*; *FRT42D*; *gCi or UAS-Ci*; $ci^{94}$/*Dp*(*1*;*4*)*1021*, $y^{+}$, $sv^{spa\text{-}pol}$ males.

To test rescue of $ci^{94}$ null animals, *ptc-lacZ*; $ci^{94}$/*Dp*(*1*;*4*)*1021*, $y^{+}$, $sv^{spa\text{-}pol}$ females were crossed to *gCi* (or derivatives); $ci^{94}$/*Dp*(*1*;*4*)*1021*, $y^{+}$, $sv^{spa\text{-}pol}$ males. Rescue in the presence of $ci^{Ce-2}$ was tested using males with this allele replacing $ci^{94}$.

To test RNAi effects in the presence of only *gCi* or *SV-1* in whole discs, *yw hs-flp*; *Sp/Cyo* ; *(gCi) (SV-1) C765 ptc-lacZ/TM6B*; $ci^{94}$/*Dp(1;4)1021*, $y^{+}$, $sv^{spa\text{-}pol}$ females were crossed to *yw hs-flp*: (*UAS-mago RNAi*) (*Sp*)/*Cyo*; (*gCi*) (*SV-1*) (*UAS-Srp54 RNAi*) (*UAS-Diap1*); $ci^{94}$/*Dp(1;4)1021*, $y^{+}$, $sv^{spa\text{-}pol}$

males. For the analogous test in *pka* mutant MARCM clones, *yw hs-flp UAS-GFP*; *tub-Gal80 FRT40A*; (*gCi* or *SV-1*) *C765 ptc-lacZ/TM6B*; $ci^{94}/Dp(1;4)1021$, $y^+$, $sv^{spa\text{-}pol}$ females were crossed to *yw hs-flp*; $pka\text{-}C1^{H2}$ *FRT40A/CyO*; (*gCi* or *SV-1*) *UAS-Diap1* (*UAS-Srp54 RNAi*); $ci^{94}/Dp(1;4)1021$, $y^+$, $sv^{spa\text{-}pol}$ males and *yw hs-flp UAS-GFP*; *tub-Gal80 FRT40A*; $Su(fu)^{LP}$ *C765 ptc-lacZ/TM6B*; $ci^{94}/Dp(1;4)1021$, $y^+$, $sv^{spa\text{-}pol}$ females were crossed to *yw hs-flp*; $pka\text{-}C1^{H2}$ *FRT40A UAS-mago RNAi/CyO*; (*gCi* or *SV-1*); $ci^{94}/Dp(1;4)1021$, $y^+$, $sv^{spa\text{-}pol}$ males.

All wings or stained wing discs shown were from male animals when mutant for *fu*; in all other cases, wing discs were dissected from larvae without sorting males from females.

### Mutagenesis and cloning

Genomic transgenes were created by cloning the entire 16-kb genomic *ci* region from a Bluescript-SK (BSK) vector [provided by K. Basler (Methot and Basler 1999)] into an att-Pacman Expression vector (*Drosophila* Genomics Resource Center). To facilitate mutagenesis, the 16-kb fragment was first separated into two parts. The region including the promoter, first exon, and part of the first intron ("Ci fragment 2") was cloned as a *Bam*HI-*Nhe*I fragment into BSK cut with *Bam*HI and *Xba*I to create BSK-CiF2. The complementary *Nhe*I-*Kpn*I fragment containing all other exons and the 3′-UTR ("Ci Fragment 1") was cloned into BSK cut with *Spe*I and *Kpn*I to create BSK-CiF1. BSK-CiF2 was cut with *Not*I and *Bsp*1201 to clone the whole CiF2 fragment into the P[acman]-$Cm^R$ vector cut with *Not*I, so that *Rsr*II and *Pme*I vector sites were downstream of *ci* first intron sequences in RP-CiF2. CiF1 was amplified from BSK-CiF1 by long-range PCR using PfuUltraII Fusion HS DNA polymerase (Agilent Technologies), adding *Rsr*II and *Pme*I at either end and cloning the product into a Zero Blunt Topo cloning vector (Invitrogen, Carlsbad, CA). The *Rsr*II-*Pme*I fragment was then cloned into RP-CiF2 cut with the same enzyme to create the final Pacman vector containing the entire 16 kb genomic ci DNA. The 28 kb gCi AttPacman transgene was then inserted at the *att ZH-86Fb* landing site at cytological location 86F8 (Rainbow Transgenic Services). To create *gCi ATG-A* and *gCi ATG-B*, ATG codons were first altered in BSK-CiF2 and BSK-CiF2, respectively, using the QuikChange II site-directed mutagenesis kit (Agilent Technologies) (primers are listed in Supplemental Material, Table S2 in File S1). To create *SV-1* and *Ci-1*, Ci coding sequences were amplified from a full-length *ci* cDNA using primers beginning 2- nt upstream of the initiation codon and at the stop codon but preceded by an added *Xba*I site. This amplicon was partially digested with *Aat*II and *Xba*I to generate a 4.2-kb coding region fragment that was cloned between *Aat*II (which cuts 23 nt upstream of the initiation codon for Ci-A) and *Xba*I sites of BSK-CiF2. The resulting DNA was cut with *Bsp*1201 and *Not*I to release an 11.5-kb fragment that was cloned into the *Not*I site of attB-P[acman]-$Ap^R$ (producing att-BP[acman]-PCi). A 1-kb segment of DNA downstream of the stop codon was amplified by PCR from BSK-CiF1, adding *Not*I and *Pac*I to either end, and cloned between the *Not*I site and *Pac*1 sites of att-BP[acman]-PCi to produce the *Ci-1* transgene. Alternatively, the 3′-UTR from SV40 was amplified from pUAST-attB with addition of *Not*I and *Pac*I sites and cloned similarly into att-BP[acman]-PCi to produce the *SV-1* transgene. The *UAS-Srp54* transgene was constructed by amplifying the coding region from the FBcI0164286 cDNA clone (DGRC) with addition of *Not*I and *Xho*I sites, followed by cloning into pUAST-attB and introduction into the 86F *att* landing site. An analogous method was used to make *UAS-CG3605*, starting from the FBcl10177075 cDNA clone (DGRC), adding *Eco*RI and *Kpn*I sites to clone it into *pUAST-attB*.

### Immunohistochemistry

Wing discs were dissected from late third instar larvae in PBS and fixed in 4% paraformaldehyde (in PBS) for 30 min, rinsed 3× with PBS, blocked with 10% normal goat serum (Jackson ImmunoResearch Laboratories) in PBST (0.1% Triton) for 1 hr, and stained with the following primary antibodies: rabbit anti-caspase 3 (1:100; D175, Cell Signaling), rabbit anti-β-galactosidase (1:10000; MP Biomedicals), mouse 4D9 anti-engrailed (1:5; Developmental Studies Hybridoma Bank), rat 2A1 anti-Ci (1:3; Developmental Studies Hybridoma Bank), and mouse anti-collier (1:10,000 from Alain Vincent, Toulouse University, France) overnight at 4°. Inverted larvae were then washed three times in PBST for 10 min each and incubated with Alexa Fluor 488, 546, 594, or 647 secondary antibodies (1:1000; Molecular Probes, Eugene, OR) for 1 hr at room temperature. Larvae were washed twice in PBST for 20 min each, once in PBS for 10 min, and mounted, with or without additional Hoechst staining (1:1000; Molecular Probes), in Aqua/Poly mount (Polysciences, Warrington, PA).

### Quantitation from fluorescent images

Fluorescence images were captured using 20×, or 63×, 1.4 NA oil immersion lenses on a confocal microscope (LSM 700; Zeiss [Carl Zeiss], Thornwood, NY). Whole wing disc images of 512 × 512 pixels at 8-bit depth were captured with a 20× lens at a resolution of 2.52 pixels/μm. Three stacks per wing disc where acquired with xzy scaling of 645, 645, and 5.3 μm. Centering of the middle stack was done based on the highest levels of *ptc-lacZ* and Ci-155 at the dorsal/ventral (D/V) boundary. The range indicator was used to set the appropriate laser intensity per experiment for each fluorophore such that the signal was in the linear range. Rotation and *z*-projection of the stacks were performed with ImageJ software (National Institutes of Health, Bethesda, MD). To measure intensity profiles along the anterior/posterior (AP) axis, an elongated rectangle was drawn on a central region of the wing pouch, avoiding the DV border. The *y*-axis shows the average fluorescence intensity over the height of the rectangle at each point on the *x*-axis (AP axis) for *ptc-lacZ* expression or Ci-155 protein, measured using Image J. At least three wings discs per condition were measured and averaged for each plot, using the posterior edge of *ptc-lacZ* expression as a reference point for the AP border. The ratio of the peak fluorescence intensity at the AP border for an experimental genotype relative to the

appropriate control processed in parallel was calculated for each experiment. These values (expressed as a percentage of controls) from at least three independent experiments were used to calculate a mean and C.I. For clones, the average fluorescent intensity over the area of the GFP-marked clone was measured using Image J. The average fluorescence intensity in anterior cells outside the clones in the same wing disc was also measured. From these measurements, the ratio of fluorescence intensities (inside/outside) was calculated for each clone. These values from multiple control and experimental clones were used to derive mean and SEM values. Representative images of clones were acquired with a 63×, 1.4 NA oil immersion lens at a resolution of 2.52 pixels/μm.

#### *Adult wings*

Adult wings were pulled off anesthetized flies and placed in 70% ethanol for 5 min, transferred to 100% ethanol, and then mounted in Aqua/Poly Mount (Polysciences). They were imaged with Transmitted Light on a Nikon Diaphot 300 microscope at 10× (Nikon, Garden City, NY).

#### *RNA analysis*

For analysis, 30–40 wing discs per genotype were dissected from third instar larvae in cold nuclease-free PBS. RNA was isolated from the wing discs using an RNeasy mini kit (QIAGEN, Valencia, CA) with DNase (QIAGEN) and converted to cDNA using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) using random primers or oligodT. Quantitative RT-PCR was performed in the StepOnePlus Real-Time PCR System with Power SYBR Green Mastermix (Applied Biosystems, Foster City, CA). Primers for amplifying each PCR product are listed in Table S3 in File S1. The relative abundance of RNAs for experimental discs *vs.* controls was calculated using the $2^{-\Delta\Delta Ct}$ method, normalizing to the housekeeping genes Rpl15 and α4-tubulin. The level of *ci-B* RNA relative to *ci-A* RNA was calculated from the Ct values for each.

#### *Statistics*

To compare experimental values to controls for fluorescence intensity (of *ptc-lacZ* expression or Ci-155 protein) measured at the AP border of wing discs, the experimental/control ratio was first calculated from individual trials using at least three wing discs of each genotype, as described earlier. These ratios from multiple trials were then used to calculate a mean value and 95% C.I. for each experimental genotype, expressed as a percentage of control values. Mean values and C.I.s of experimental samples expressed as a percentage of controls were calculated analogously for RNA measurements after converting raw quantitative (q)RT-PCR results into an experimental/control ratio for each trial. For measurements of fluorescent intensity in clones, the average clone intensity relative to surrounding anterior cells was first calculated for experimental and control samples, as described earlier. The set of values obtained from multiple clones of each genotype were examined by a Shapiro–Wilk test for a fit to a normal distribution. Mean values for experimental and control clones were then calculated and a *P*-value for the significance of differences between them was calculated using an unpaired Student's *t*-test for unequal variances. For each experiment, the number of samples and trials are given in the figure legend. The number of samples or trials was not predetermined; we aimed to test as many samples as possible (some required genotypes were rare among progeny). No samples were excluded for reasons other than poor staining or physical damage during processing.

#### *Data availability*

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully in the article. *Drosophila* stocks and other reagents are available upon request.

## Results

#### *EJC and splicing factors identified in a screen for modifiers of Hh pathway activity*

Hh is expressed in posterior compartment cells of the wing disc and is transported to a strip of neighboring anterior cells to induce transcription of several patterning genes at the AP border (Vervoort 2000; Xiong *et al.* 2015). These Hh target genes include *ptc*, conveniently reported by the level of *ptc-lacZ* expression, and *col* (Figure 1, G and H), which is essential for specifying the tissue between the central two veins of the mature wing. To identify new contributors to Hh signaling pathway activity, we tested a large set of heterozygous autosomal deficiencies for the ability to modify the wing phenotypes of flies heterozygous for *Su(fu)* and lacking Fu kinase activity; these flies have reduced intervein territory between veins 3 and 4 (v3–4, Figure 1, A and B) resulting from impaired Hh signaling. Only two deficiencies, including *Su(fu)* and *cos2*, respectively, increased v3–4 separation, consistent with enhanced Hh pathway activity (Table S1 in File S1). For each deficiency that narrowed the v3–4 interval, we tested several additional deletions to define a critical region. Ten intervals with at least two overlapping deficiencies narrowing v3–4 were identified from a total of ∼220 deficiencies successfully screened (Table S1 in File S1).

The phenotype of one deficiency was reproduced by a point mutation in the *tsunagi* (*tsu*; aka *Y14*) gene (Figure 1C). Because Y14 and Mago nashi (Mago) are core members of the EJC, we also tested a null mutation of *mago* as a modifier and found that it also narrowed v3–4 spacing (Figure 1D). This spacing could theoretically be altered by reducing Hh pathway activity or by affecting downstream steps. The latter mechanism is likely for a set of overlapping deficiencies that included *spalt* and *spalt-related*, which have established roles in vein specification (Organista *et al.* 2015) (Table S1 in File S1). The MAPK *rolled* is also involved in vein formation and is a known target of Mago and Y14 (Blair 2007; Ashton-Beaucage *et al.* 2010; Roignant and Treisman 2010). However, the *mago* phenotype was not reversed by ectopic expression of a *UAS-MAPK* transgene shown previously to rescue MAPK

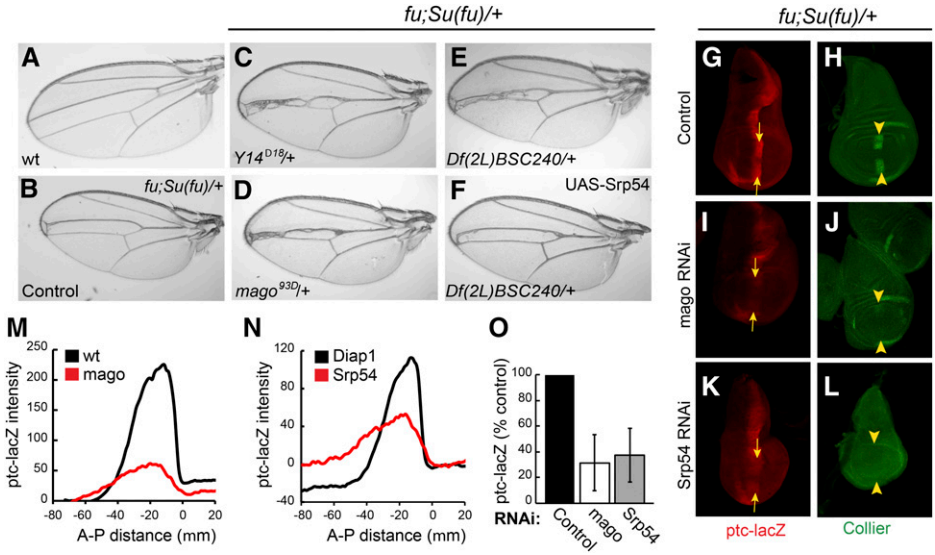


**Figure 1** Screen for Hh pathway modifiers. (A–F) Wings from (A) wild-type males and (B–F) *fu*; *Su(fu)*/+ males. (B) Narrowing of the central veins was increased by heterozygosity for (C) $Y14^{\Delta 18}$ and (D) $mago^{93D}$. Narrowing due to (E) *Df(2L) BSC240* was (F) largely suppressed by expression of *UAS-Srp54* cDNA with *C765-GAL4*. (G and H) Wing discs from *fu*; *Su(fu)*/+ larvae have reduced expression at the AP border (yellow arrows) of (G) *ptc-lacZ* (red) and (H) Collier [green; restricted to the wing pouch (arrowheads)] relative to wild-type (data not shown). (I–L) Both *ptc-lacZ* and Collier were reduced further by *C765-GAL4*-driven expression of (I and J) *UAS-mago RNAi* or (K and L) *UAS-Srp54 RNAi* together with *UAS-Diap1* (*UAS-Diap1* was always used as a control for this genotype). Reduced *ptc* induction limits Ptc-induced endocytosis of Hh, so a weaker *ptc-lacZ* AP border stripe is also generally broader. (M and N) Average *ptc-lacZ* intensity along the AP axis (anterior to the left of zero) of the wing pouch for four wing discs in a single experiment, comparing (M) *UAS-mago RNAi* to control and (N) *UAS-Srp54 RNAi* to control (both also express *UAS-Diap1*). (O) Maximal *ptc-lacZ* intensity at the AP border (derived from profiles along the AP axis) as a percentage of controls for discs expressing *mago* and *Srp54* RNAi, showing means and 95% C.I.s ($n = 4$ experiments for *mago RNAi* and $n = 3$ experiments for *Srp54 RNAi*). AP, anterior/posterior; Hh, Hedgehog; Ptc, Patched; RNAi, RNA interference; wt, wild-type.

function impaired by EJC depletion (Roignant and Treisman 2010) (Figure S1, D and E in File S1). Thus, Mago is not acting via MAPK and may instead be affecting the Hh pathway.

To identify the key gene within two additional narrowly defined deletion intervals, we tested the consequence of expressing all relevant available *UAS-RNAi* transgenes in a sensitized (*fu*; *Su(fu)*/+) background using *C765-GAL4*, which is expressed selectively in wing discs. RNAi directed toward the genes *CG4602* (*Srp54*) and *CG3605* did not permit eclosion of any adults but significantly reduced the activity of the universal Hh pathway reporter, *ptc-lacZ*, at the AP border of third instar larval wing discs (Figure S2C in File S1 and data not shown). However, both also variably reduced the size of wing discs, disrupted nuclear integrity, and induced activated caspase 3 staining, indicative of apoptosis (Figure S2C' in File S1 and data not shown). Coexpression of the antiapoptotic *Diap1* gene largely rescued these phenotypes for *Srp54 RNAi* (Figure S2, A'–D' in File S1) but less completely for RNAi toward *CG3605*. In both cases, there was still a marked reduction in *ptc-lacZ* staining at the AP border of wild-type or *fu*; *Su(fu)*/+ wing discs (Figure 1, G, K, N, and O and Figures S1, F–M and S2, A–D in File S1). Expression of *UAS-Srp54* using *C765-GAL4* largely reversed the v3–4 narrowing of *fu*; *Su(fu)*/+ flies due to *Df(2L) BSC240* (Figure 1, E and F), while *UAS-CG3605* partially suppressed v3–4 narrowing due to *Df(2L) Exel7014* (Figure S1, A–C in File S1), suggesting that *Srp54* and *CG3605* were critical modifier genes within the two deficiencies that affected Hh pathway activity. Srp54 belongs to the family of SR proteins, which contain RNA-binding and Arg/Ser-rich domains, and are generally involved in RNA splicing (Bradley *et al.* 2015). Both Srp54 and *CG3605* have been identified in spliceosomal complexes and functionally implicated in RNA splicing (Kennedy *et al.* 1998; Park *et al.* 2004; Wu *et al.* 2006; Herold *et al.* 2009).

Collier is induced at the AP border of wing discs by moderately high levels of Hh signaling (Vervoort 2000) and consequently has lower expression in a *fu*; *Su(fu)*/+ background than in wild-type wing discs. Expression of Collier was eliminated by additional expression of *Srp54* or *CG3605 RNAi* transgenes (Figure 1, H, J, and L and Figure S1, F' and H' in File S1). Likewise, *mago RNAi* eliminated Collier induction at the AP border of *fu*; *Su(fu)*/+ wing discs and strongly reduced *ptc-lacZ* expression (Figure 1, G–J, M, and O), directly implicating Mago in Hh signaling. Expression of *mago RNAi* did not induce cell death or other morphological phenotypes. Because the apoptotic phenotype of *CG3605* inhibition was not fully suppressed by Diap1, we focused further studies on just Srp54 and Mago, and always expressed excess Diap1 together with *Srp54 RNAi* to suppress cell death.

### *Mago and Srp54 alter Ci-155 levels*

The effects of reducing Mago and Srp54 activity on Hh pathway activity and the levels of Ci-155 were measured in otherwise wild-type wing discs. Mago inhibition lowered Ci-155 levels at the AP border and in anterior regions by ~30% (Figure 2, G, H, K, and M). RNAi transgenes directed to two other core pre-EJC components, Y14 and eIF4AIII, also reduced Ci-155 levels in wild-type wing discs and reduced *ptc-lacZ* expression in wing discs with impaired Hh signaling (*fu*; *Su(fu)*/+) (Figure S3 in File S1). Hh pathway activity, measured by expression of *ptc-lacZ* and the high-level Hh target gene, Engrailed (En) at the AP border, was not inhibited by RNAi directed to Mago, Y14, or eIF4AIII in wild-type wing discs (Figure 2, A, B, D, and F and data not shown). Inhibition of Barentsz (Btz), which is a largely cytoplasmic core EJC component (MLN51 in mammals), did

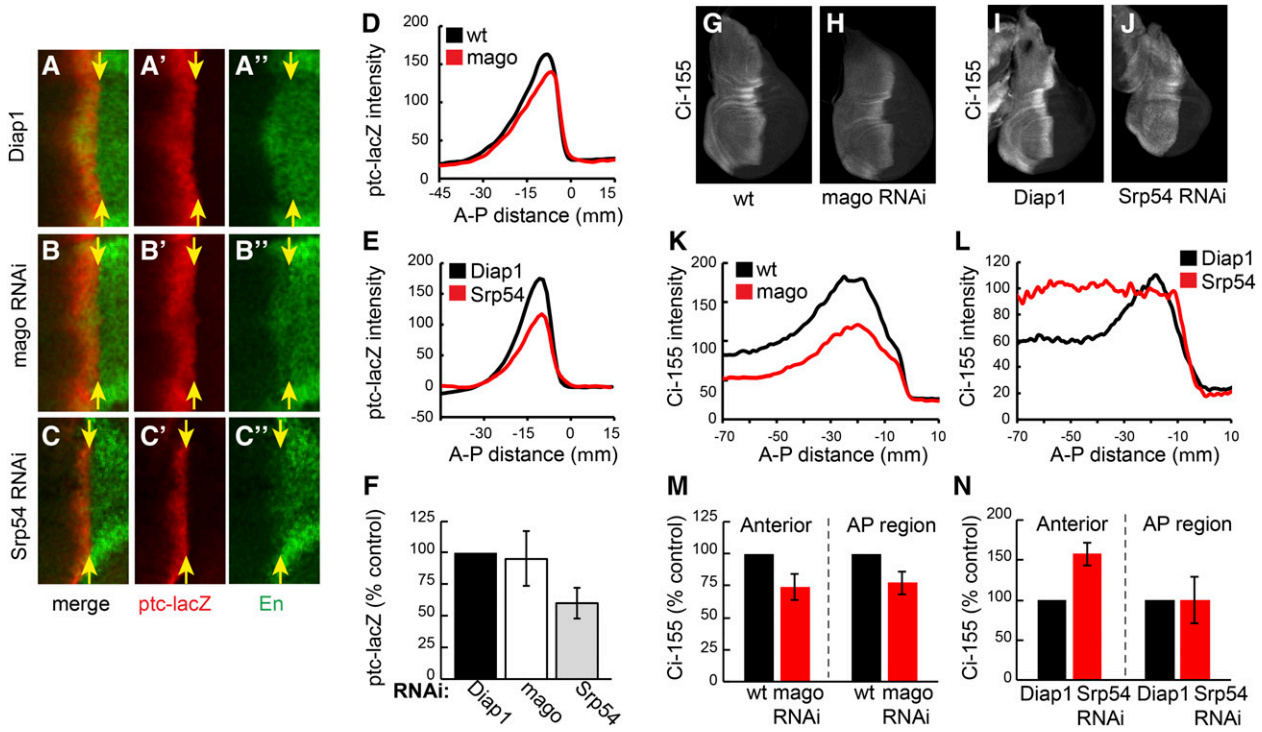


**Figure 2** Different effects of Mago and Srp54 inhibition on Ci-155 levels and activity. (A–C) Central region (around DV and AP borders) of wing discs expressing (A) *UAS-Diap1*, (B) *UAS-mago RNAi*, and (C) *UAS-Srp54 RNAi* plus *UAS-Diap1* under the control of *C765-GAL4*, stained for *ptc-lacZ* (red) and Engrailed (En; green). Only Srp54 RNAi reduced *ptc-lacZ* expression and prevented Hh induction of anterior En (to the left of yellow arrows indicating the posterior edge of the AP border). (D and E) *ptc-lacZ* intensity profiles along the AP axis for (D) *UAS-mago RNAi* and (E) *UAS-Srp54 RNAi* compared to controls for a single experiment (average of 3–4 discs). (F) Maximal *ptc-lacZ* intensity as a percentage of controls for discs expressing *mago* and *Srp54 RNAi*, showing means and 95% C.I.s ($n$ = 6 experiments for *mago RNAi* and $n$ = 3 experiments for *Srp54 RNAi*). (G–J) Wing discs expressing (H) *UAS-mago RNAi*, (J) *UAS-Srp54 RNAi*, and (G and I) controls, stained for Ci-155 (white). (K–L) Ci-155 intensity profiles along the AP axis for single experiments (average of four wing discs), comparing (K) Mago and (L) Srp54 inhibition to controls. (M and N) Maximum Ci-155 intensity in anterior and AP border cells as a percentage of controls for discs expressing *mago* and *Srp54* RNAi, showing means and 95% C.I.s ($n$ = 5 experiments for *mago RNAi* and $n$ = 4 experiments for *Srp54 RNAi*). AP, anterior/posterior; DV, ; Hh, Hedgehog; Ptc, Patched; RNAi, RNA interference; wt, wild-type.

not alter *ptc-lacZ* expression in *fu*, *Su(fu)/+* wing discs or Ci-155 levels (Figure S3, D, E, J, and K). A nuclear function of Mago, Y14, and eIF4AIII that is not shared by Btz has been demonstrated for normal splicing of *MAPK* and *piwi* in *Drosophila* (Roignant and Treisman 2010; Ashton-Beaucage and Therrien 2011; Hayashi *et al.* 2014; Malone *et al.* 2014). Therefore, we hypothesized that Mago, Y14, and eIF4AIII core EJC components affect Hh pathway activity under sensitized conditions by acting directly on *ci* RNA splicing to reduce Ci-155 protein levels.

Reduction of Srp54 activity also altered the profile of Ci-155 in wing discs but in a different manner. Ci-155 levels were not significantly altered at the AP border but were higher throughout the rest of the anterior compartment compared to wild-type discs (Figure 2, I, J, L, and N). Inhibition of Srp54 also reduced *ptc-lacZ* expression and almost eliminated anterior En expression at the AP border of wild-type wing discs (Figure 2, A, C, E, and F). The changes in Ci-155 levels and *ptc-lacZ* expression due to *Srp54 RNAi* were suppressed by expression of an *Srp54* cDNA (Figure S2, E–H), confirming attribution to Srp54. The observed Ci-155 profile suggests that *Srp54 RNAi* may impair Ci-155 processing in anterior cells, so that Ci-155 levels cannot reliably report any additional changes in *ci* RNA.

### *Effects of Mago and Srp54 inhibition on ci RNA*

The most direct way that Mago might alter Ci-155 levels is by acting on *ci* RNA. The major documented *ci* RNA is denoted *ci-A* but a much less prevalent RNA, *ci-B*, has been deduced from a small number of cDNAs and RNA-seq data (FlyBase). Those data suggest that *ci-B* RNA initiates at a site upstream of *ci-A* and uses an alternative splice donor at the end of the first exon that is just 8 nt downstream of the *ci-A* 5′-end (Figure 3A). Using RNA from wild-type wing discs we were able to detect a product of the expected size using primers corresponding to exon 1B and exon 2, but at much lower levels than for an exon 1A to exon 2 primer pair (Figure 3B). These data confirm the presence of *ci-B* RNA in wing discs. The absence of a larger product corresponding to the E1A splice donor when using primer 1B supported the idea that RNAs initiating upstream of the E1B primer use only the E1B donor splice site. qRT-PCR experiments with the same primer pairs indicated that *ci-A* is roughly 200-fold more abundant than *ci-B* in wing discs (difference between Ct values was 7.5 ± 0.64).

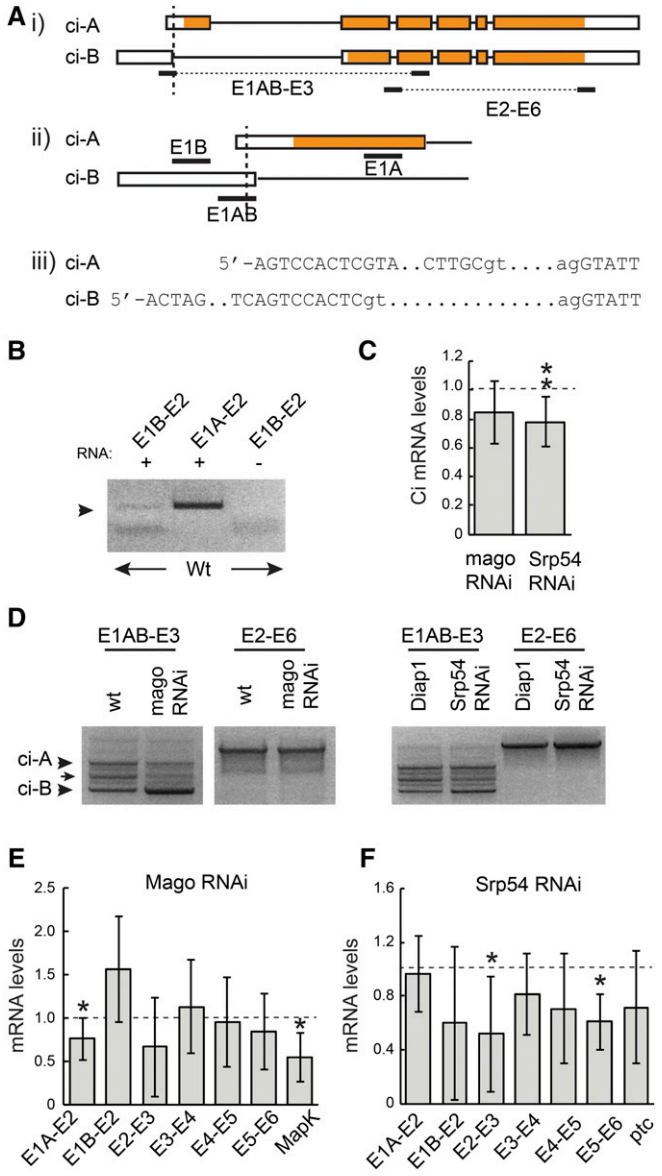


**Figure 3** Mago and Srp54 regulate *ci* RNA. (A) Schematic representation of *ci-A* and *ci-B* RNAs, together with primer locations (exons are boxed with translated segments in orange). The TSS of B is 742 nt upstream of TSS-A. *ci-B* first exon donor splice site is 8 nt downstream of TSS-A. *ci-A* first exon is 469 nt and first intron is 3446 nt; *ci-B* first exon is 751 nt and first intron is 3906 nt. (B) RT-PCR products from wild-type wing disc RNA using the indicated primer pairs. (C) *ci* RNA levels as a fraction of controls (set at 1.0; dashed line) measured by qRT-PCR with primers from exon 3 and spanning the exon 2/3 junction for total RNA from wing discs expressing *mago RNAi* or *Srp54 RNAi*. (D) RT-PCR products obtained using the indicated primer pairs using RNA from wing discs with reduced Mago, Srp54, or their controls. Products of the expected sizes for *ci-A* and *ci-B* (arrowheads) were confirmed by sequencing. An intermediate band (arrow) revealed a clear product on one occasion, corresponding to splicing between the *ci-B* donor site and an acceptor 277 nt downstream, followed by splicing in the *ci-A* pattern (and therefore encoding the normal Ci-A protein product). (E and F) RNA levels as a fraction of controls (set at 1.0; dashed line) measured by qRT-PCR using primers spanning each exon junction of *ci* and exons 6–7 of MAPK for wing discs expressing (E) *UAS-mago RNAi* or (F) *UAS-Srp54 RNAi* compared to controls. (C, E, and F) Values in all qRT-PCR experiments were first normalized to Rp49 and Rpl45 levels. Means and 95% C.I.s are shown relative to controls (set at 1.0) for (C) $n = 9$ experiments and (E and F) $n = 3$ experiments for all except E1A–E2 for *mago RNAi* and *Srp54 RNAi* ($n = 7$), E1B–E2, E2–E3, E4–E5, and E5–E6 for *mago RNAi* ($n = 4$), MAPK ($n = 5$), and E5–E6 for *Srp54 RNAi* ($n = 4$). Significant differences to controls were also calculated by Student's *t*-test (* $P < 0.05$, ** $P < 0.01$). qRT-PCR, quantitative RT-PCR; RNAi, RNA interference; TSS, transcription start site; wt, wild-type.

We then explored whether inhibition of Mago or Srp54 altered the amounts or splicing patterns of these *ci* RNAs.

We observed reduced levels of *ci* RNA by qRT-PCR using primers that amplified sequences in exon 3 for both *mago* and *Srp54 RNAi* treatments of wing discs (Figure 3C). To compare *ci-A* and *ci-B* RNA levels, while selectively increasing detection sensitivity for *ci-B* RNA we used a primer (E1AB) that matched exon 1B throughout its 20 nt but matched exon 1A only at the last 9 nt. Using this primer together with an exon 3 primer for RT-PCR revealed three clear bands (Figure 3D). Two were sequenced and found to correspond to the expected sequences of *ci-A* and *ci-B* in this region. The ratio of these two bands was not discernibly altered in wing discs expressing *Srp54 RNAi* but Mago inhibition significantly increased the *ci-B* product relative to *ci-A* (Figure 3D). No novel RNA splicing patterns or included introns were detected in response to Mago or Srp54 inhibition for regions spanning exons 1–3 or 2–6 (Figure 3D). qRT-PCR measurements supported the inference that RNA with the splice characteristic of *ci-B* was increased, while RNA with the *ci-A* splice was reduced in response to Mago inhibition (Figure 3E).

RNA spliced across introns common to *ci-A* and *ci-B* was reduced by *mago RNAi* in some cases, but not consistently (Figure 3E). A known Mago target in the *MAPK* gene was used as a positive control (Roignant and Treisman 2010) and showed reduced spliced product, as expected. The alteration in the pattern of *ci* RNAs suggests that Mago may act directly on *ci* splicing. Loss of Mago may be affecting the choice of splice donor sites for the first exon to allow some E1B donor use for the major *ci* primary transcript. Alternatively, loss of Mago might be affecting the choice of transcription start sites, increasing initiation at the upstream site characteristic of *ci-B* and decreasing initiation from the major *ci-A* start site. In either case, the reduced levels of E1A–2 spliced RNA observed (Figure 3, D and E) would be expected to decrease Ci-155 protein levels, while increased *ci-B* RNA levels would only produce a very small increase in protein product because this RNA is present at only ~1% of *ci-A* RNA levels.

Inhibition of Srp54 led to a reduction in spliced RNA across all main-body introns but, in contrast to Mago inhibition, it did not increase E1B–E2 spliced RNA or significantly reduce E1A–E2 spliced RNA levels (Figure 3, D and F). *ptc* RNA levels were reduced (Figure 3F), consistent with reduced *ptc-lacZ* expression at the AP border. The reduced amount of *ci* RNA measured across all constitutive splice junctions would be expected to result in reduced levels of Ci-155 primary translation product.

### *Cell autonomy of Mago and Srp54 effects on Hh pathway activity*

If EJC proteins or Srp54 affect Hh signaling by acting on *ci* RNA, we would expect them to act in cells responding to Hh,

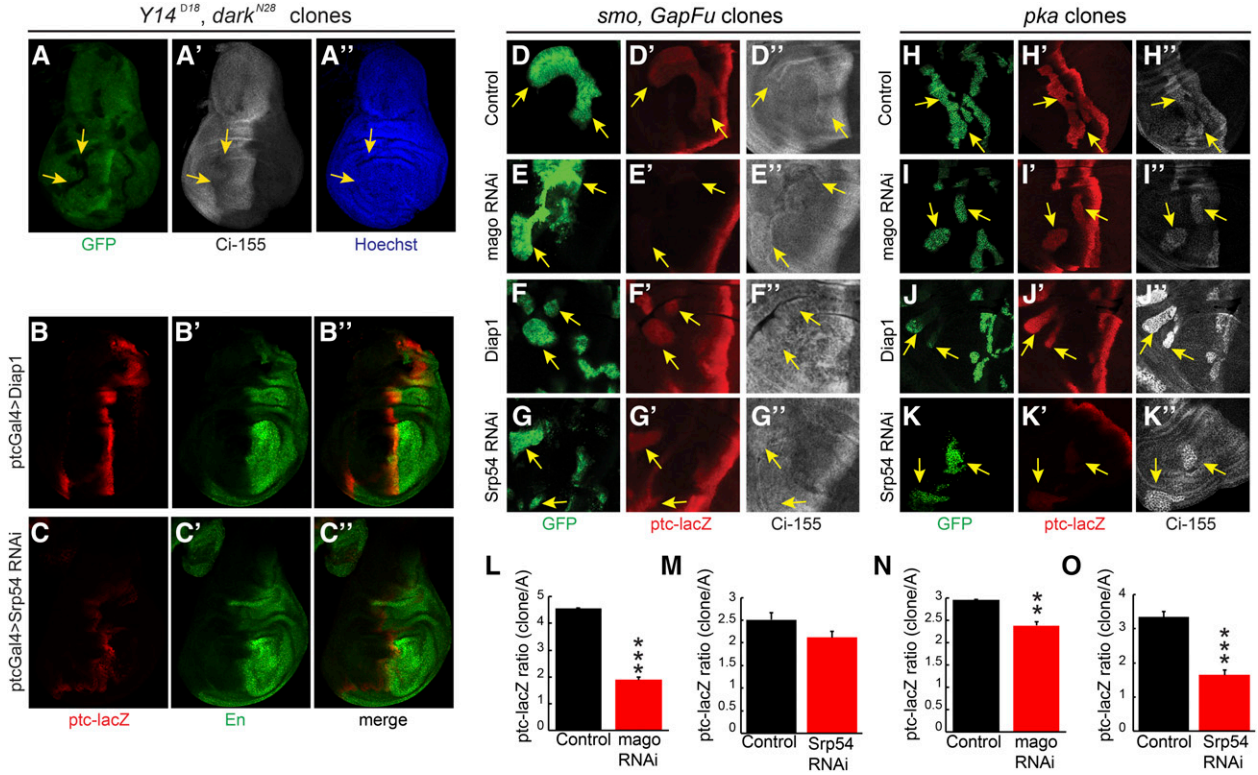


**Figure 4** Cell autonomous action of EJC members and Srp54 on Hh pathway activity. (A) Homozygous $Y14^{\Delta 28}$ $dark^{N28}$ clones (arrows) in a *Minute* background, marked by loss of GFP (green), had lower Ci-155 levels [white, (A′)] but no cell death or rearrangement evident from nuclear Hoechst staining [blue, (A″)]. (B–C) Expression of *UAS-Srp54 RNAi* with *ptc-GAL4* driver reduced *ptc-lacZ* (red) and anterior En (green) expression relative to *UAS-Diap1* controls. (D–G) Ectopic *ptc-lacZ* (red) was induced in anterior *smo* clones expressing *UAS-GapFu* (marked by GFP, green, arrows) but induction was reduced (E) greatly by *UAS-mago RNAi* and (G) slightly by *UAS-Srp54 RNAi*. (D″–G″) Changes in Ci-155 levels (white) were small. (H–K) Ectopic *ptc-lacZ* (red) was induced in anterior *pka* clones (marked by GFP, green, arrows) but induction was reduced (I) slightly by *UAS-mago RNAi* and (K) greatly by *UAS-Srp54 RNAi*. (H″–K″) The increase of Ci-155 (white) was clearly reduced by Srp54 RNAi in some *pka* clones. (L–O) *ptc-lacZ* intensity relative to controls within (L and M) *smo GapFu* clones or (N and O) *pka* clones, showing mean, SEM, and significant differences calculated by Student's *t*-test (** $P < 0.001$, *** $P < 0.0001$), using (L) $n = 4$ control and $n = 8$ experimental clones, (M) $n = 13$ control and $n = 21$ experimental clones, (N) $n = 21$ control and $n = 18$ experimental clones, and (O) $n = 15$ control and $n = 16$ experimental clones. EJC, exon junction complex; En, engrailed; Hh, Hedgehog; Ptc, Patched; RNAi, RNA interference; wt, wild-type.

rather than in cells responsible for producing or transporting Hh. Strong alleles of *Y14* or *mago* did not readily produced large homozygous mutant wing disc clones but we did observe a cell autonomous reduction of Ci-155 levels for *Y14* clones when clone survival was enhanced by using a *Minute* background (Amoyel and Bach 2014) and a mutant allele of the proapoptotic *dark* gene (Roignant and Treisman 2010) (Figure 4A). We also observed a reduction in AP border *ptc-lacZ* and En expression when *Srp54 RNAi* expression was limited to anterior cells by using *ptc-GAL4* as a driver (Figure 4, B and C). However, the most convincing evidence of Mago and Srp54 acting in Hh signal transduction was observed by creating MARCM clones (Lee and Luo 2001) that expressed the corresponding *RNAi* in anterior cells and had ectopic Hh pathway activity due to genetic alteration of PKA or Fu activity.

Reduced *ptc-lacZ* expression due to *mago RNAi* was clearest in clones expressing activated Fu elicited by expression of GAP-Fu, a membrane-tethered Fu fusion protein containing the myristoylation domain from Growth-Associated-Protein-43 (Claret *et al.* 2007; Zhou and Kalderon 2011) (reduced 60%, Figure 4, D, E, and L). Reduced *ptc-lacZ* expression was also evident in *pka* (Figure 4, H, I, and N) and *cos2* (Figure S4, G, H, and K in File S1) mutant clones (reduced 20% in each case). A cell autonomous reduction of *ptc-lacZ* expression due to Srp54 inhibition was also seen for the same three types of clone. The effects of Srp54 inhibition were larger for *pka* mutant clones (reduced 50%, Figure 4, J, K, and O) than for *cos2* mutant clones (reduced ~20%, Figure S4, I, J, and L in File S1) or for clones with activated Fu (reduced 15%, Figure 4, F, G, and M). No significant reduction of *ptc-lacZ* due to Mago or Srp54 inhibition was observed in *ptc* mutant clones (Figure S4, A–F in File S1), which have very high levels of Hh pathway activity. These results are consistent with cell autonomous actions of Mago and Srp54, which contribute significantly to Hh pathway activity when the pathway is not maximally activated (as in *pka*, *cos2*, and *GAP-Fu* mutant clones and at the AP border of discs lacking Fu kinase activity).

We also observed that Ci-155 levels were reduced by *Srp54 RNAi* in *pka* mutant clones (Figure 4, J" and K"). There is no

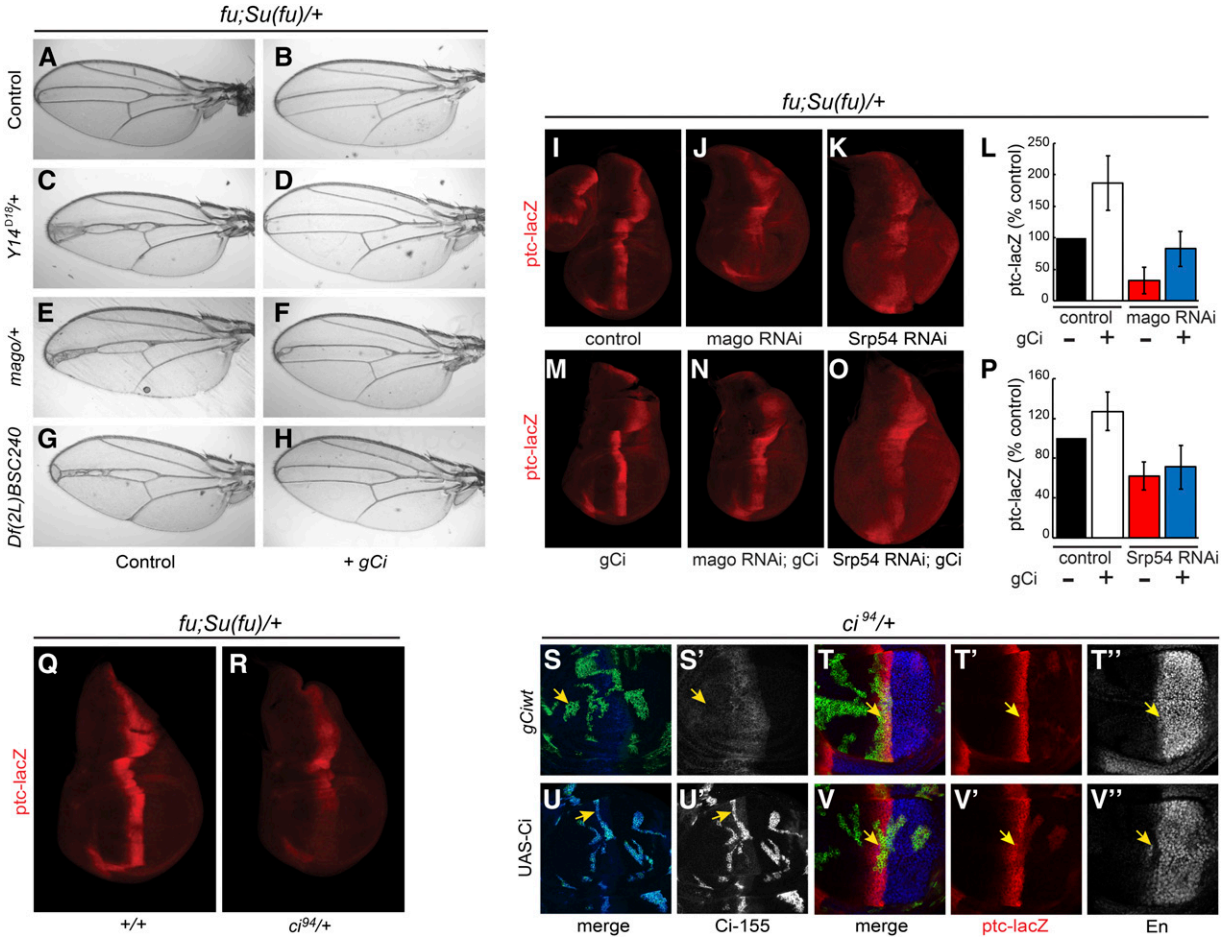


**Figure 5** Dependence of Hh signaling on Ci-155 levels. (A–H) Narrowing of veins 3–4 in (A) *fu*; *Su(fu)*/+ controls is increased by heterozygosity for (C) *Y14*, (E) *mago*, or (G) *Df(2L)BSC240*, but these changes were (B, D, F, and H) suppressed by addition of a single copy of the *gCi* transgene. (I–P) In *fu*; *Su(fu)*/+ wing discs a single copy of the *gCi* transgene (I and M) increased *ptc-lacZ* expression and (J–O) suppressed inhibition of *ptc-lacZ* expression by (J and N) *mago RNAi* but (K and O) not by *Srp54 RNAi*. (L and P) Maximal *ptc-lacZ* intensity as a percentage of controls for *fu*; *Su(fu)*/+ wing discs expressing *mago* and *Srp54* RNAi, with or without a *gCi* transgene, showing means and 95% C.I.s for $n = 4$ experiments. (Q and R) Loss of one copy of *ci* (R) reduced *ptc-lacZ* (red) expression in *fu*; *Su(fu)*/+ wing discs. (S–V) MARCM clones (marked by GFP, green) that lose a second chromosome *gCi* transgene in wing discs that are $ci^{94}$/+ and either (S and T) include a third chromosome *gCi* transgene or (U and V) express *UAS-Ci* with *C765-GAL4*. (S and U) Ci-155 levels (white) were increased greatly by *UAS-Ci* expression but were not affected by exchange of *gCi* transgenes. (T and V) Only excess Ci-155 from *UAS-Ci* decreased *ptc-lacZ* (red) and anterior En (white) expression at the AP border (yellow arrows). AP, anterior/posterior; En, engrailed; Hh, Hedgehog; Ptc, Patched; RNAi, RNA interference; wt, wild-type.

Ci-155 processing in the absence of PKA, so the reduced levels of Ci-155 likely represent a lower rate of Ci-155 production, consistent with the observation that Srp54 RNAi reduced *ci* RNA levels (Figure 3).

The additional effect of Srp54 RNAi reducing the rate of Ci-155 processing, inferred from elevated anterior Ci-155 in otherwise normal wing discs (Figure 2, J, L, and N), can explain the different impacts of Mago and Srp54 in different clones. Hh target gene activation appears to be more sensitive to reductions in Ci-155 levels in *GAP-Fu* clones than in *pka* clones based on the stronger effect of Mago inhibition in *GAP-Fu* clones. However, the reduction of Ci-155 levels expected from *Srp54 RNAi* lowering *ci* RNA is substantially offset in *GAP-Fu* clones (and not at all in *pka* clones) because *Srp54 RNAi* additionally reduces Ci-155 processing only in the *GAP-Fu* clones. Hence, *Srp54 RNAi* barely inhibits *ptc-lacZ* in *GAP-Fu* clones, whereas *ptc-lacZ* inhibition in *pka* clones is greater than for *mago RNAi*, probably because *Srp54 RNAi* causes a larger reduction in *ci* RNA (Figure 3). Altogether, clonal analyses are consistent with the hypotheses that both Mago and Srp54 inhibition reduce Hh pathway activity by reducing *ci* RNA levels, and that Srp54 inhibition also impairs Ci-155 processing.

### *Effects of Ci-155 levels on Hh pathway activity*

We next explored whether reduced levels of Ci-155 primary translation product, inferred from studies of *ci* RNA and Ci-155 antibody staining, could plausibly explain the alterations in Hh pathway activity seen in response to inhibition of Mago and Srp54. In a *fu*; *Su(fu)*/+ background, heterozygosity for *Srp54* (in *Df(2L)BSC240*), *mago*, or *Y14* reduced v3–4 spacing. Normal spacing was restored by a single copy of a 16-kb *ci* genomic transgene (Methot and Basler 1999) inserted at an

**Table 1 Rescue to adulthood of $ci^{94}/ci^{94}$ null flies by *gCi* transgenes**

| | *transgene/+*; $ci^{94}/ci^{94}$ (% *transgene/+*; $ci^{94}/+$) | *transgene/transgene*; $ci^{94}/ ci^{94}$ (% *transgene/+*; $ci^{94}/+$) |
|---|---|---|
| *gCi* | 49 (*n = 298*) | 94 (*n = 1052*) |
| *gCi ATG-B* | 83 (*n = 550*) | 99 (*n = 530*) |
| *gCi ATG-A* | 0 (*n > 300*) | 0 (*n > 300*) |
| *SV-1* | 23 (*n = 699*) | 75 (*n = 350*) |
| *Ci-1* | 0 (*n = 298*) | 9 (*n = 603*) |

*att* site on chromosome 3 ("*gCi*") (Figure 5, A–H). In the same genetic background (*fu*; *Su*(*fu*)/+), addition of the *gCi* transgene enhanced *ptc-lacZ* expression (Figure 5, I, L, and M), while heterozygosity for *ci* reduced *ptc-lacZ* expression (Figure 5, Q and R). Thus, reduced *ci* gene dosage can phenocopy the effects of reduced Mago or Srp54 activity and an extra *ci* transgene can suppress heterozygous Mago and Srp54 phenotypes, consistent with Mago and Srp54 acting through regulation of *ci* RNA levels.

When Mago activity was reduced more drastically using RNAi in *fu*; *Su*(*fu*)/+ discs, adding the *gCi* transgene substantially restored *ptc-lacZ* expression (Figure 5, J, L, and N), consistent with Mago acting solely by modifying *ci* RNA levels. However, analogous complementation was not observed for *Srp54 RNAi* (Figure 5, K, O, and P), suggesting that severe reduction in Srp54 activity does not act solely by reducing *ci* RNA.

The effect of excess Ci on Hh signaling was tested by expressing a *UAS-Ci cDNA* transgene using *C765-GAL4* in MARCM clones at the AP border. Ci-155 levels were greatly elevated in clones and induced strong *ptc-lacZ* expression in posterior clones, as expected due to stimulation by Hh (Smelkinson *et al.* 2007), but did not induce *ptc-lacZ* in anterior clones (Figure 5, S–V). Surprisingly, clones at the AP border showed reduced expression of both *ptc-lacZ* and En (Figure 5V), whereas control clones had no such changes (Figure 5T), implying that excess Ci-155 can impair Hh signaling. Thus, small reductions in Ci-155 primary translation product can reduce Hh pathway activity under sensitized conditions, while excess Ci-155 can impair normal Hh signaling.

### *ci-A and ci-B RNA encode similar Ci activator functions*

The confirmed existence of *ci-B* RNA in wing discs, albeit at much lower levels than *ci-A* RNA, as well as the potential for Mago to regulate the relative levels of *ci-A* and *ci-B* (Figure 3), led us to question the functional role of *ci-B* RNA. The *ci-B* RNA does not include the first coding exon of *ci-A* and it would be expected to encode a translation product that initiates at M119 of the Ci-A protein (Figure 3A). Therefore, we constructed two variants of the *gCi* transgene, in which the expected initiation codons for either Ci-A (*gCi ATG-A*) or Ci-B (*gCi ATG-B*) were altered to AAG Lys codons.

We found that *gCi ATG-B* (lacking Ci-B) rescued *ci* null ($ci^{94}$) flies to adulthood with a similar efficiency to *gCi WT* (Table 1); the profile of *ptc-lacZ* expression and Ci-155 protein of rescued wing discs was also very similar (Figure 6, A–D), suggesting that Ci-B protein has no essential function under normal conditions. It remains possible that *ci-B* RNA has a function independent of protein products or that it encodes a protein initiating downstream of M119 that substitutes for Ci-B function when M119 is altered.

Neither one nor two copies of *gCi ATG-A* rescued any *ci* null animals to adulthood (Table 1), but rescue to third larval instar was observed. Wing discs from those animals showed reduced levels of AP border *ptc-lacZ* and Ci-155, together with enlarged anterior compartments (Figure 6, E–H). Ci-B (the sole expected product of *gCi ATG-A*) may have little or no Ci-75 repressor function based on the prior observation of loss of repressor activity for a Ci variant lacking residues 6–339 (Zhou and Kalderon 2010). Loss of Ci repressor leads to ectopic *dpp* induction in anterior cells and anterior expansion (Methot and Basler 1999). Consistent with the possibility that Ci-B defects stem largely from the failure to produce a repressor, *gCi ATG-A* was able to rescue adult flies transheterozygous for $ci^{94}$ and the $ci^{Ce}$ allele, which produces only a Ci repressor that is not regulated by Hh (Methot and Basler 1999) (Table 2). Rescue of $ci^{Ce}/ci^{94}$ animals by a single copy of *gCi ATG-A* was less efficient than for *gCi WT* and resulted in more severe narrowing of the v3–4 interval, but both shortcomings were largely rectified by providing two copies of the transgene (Figure 6, O–V and Table 2). These properties suggest that *gCi ATG-A* generates a Ci activator that is regulated normally but present at slightly reduced levels, most likely because of less efficient use of the Ci-B translation initiation codon compared to Ci-A, or perhaps reduced stability of Ci-B protein compared to Ci-A.

### *Properties of intronless ci transgenes*

It is possible that regulation of the efficiency of *ci-A* RNA splicing, or even a regulatory role of *ci-B* RNA independent of Ci-B protein production, are important for Hh signaling. To test these ideas, we constructed two *gCi* transgene variants, in which all intron sequences had been removed. One variant (*Ci-1*) retained the normal *ci* 3′-UTR sequences, while the other (*SV-1*) instead included 3′-UTR sequences from Simian Virus 40, with the expectation that this 3′-UTR, commonly employed for high-level gene expression, might enhance protein translation (Figure 6I).

*Ci-1* rescued *ci* null adults only when present in two copies, and very inefficiently, while *SV-1* had rescue activity intermediate between *Ci-1* and *gCi WT* (Table 1). Ci-155 protein levels encoded by *Ci-1* were much lower than for

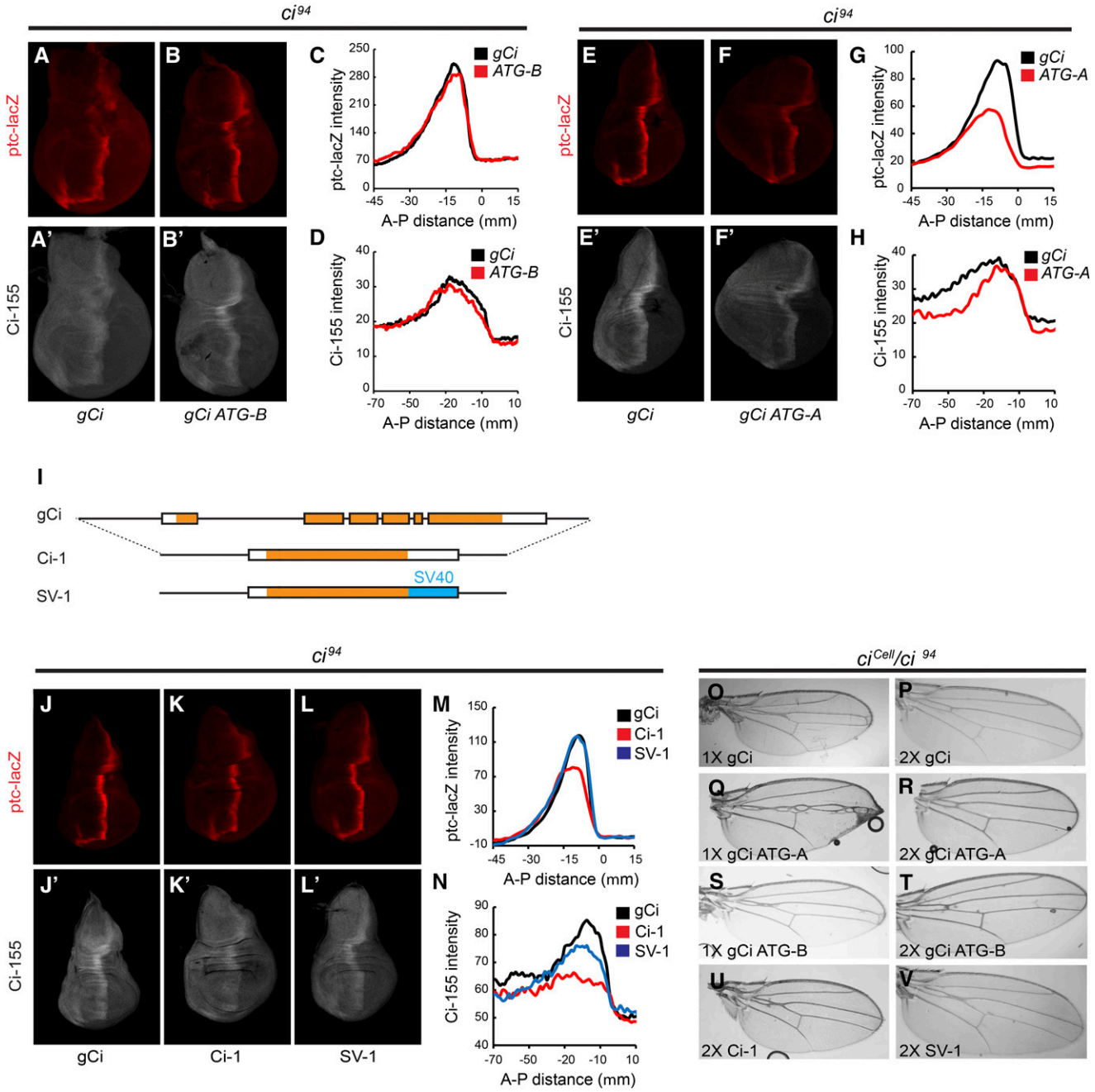


**Figure 6** Activities of Ci-A, Ci-B proteins, and intronless *ci* transgenes. (A, B, H, and F) Wing discs from *ci* null larvae with a *gCi* transgene that (A and E) is wild-type, or lacks the initiator codon for (B) Ci-B or (F) Ci-A, stained for *ptc-lacZ* (red) or Ci-155 (white). (C and G) *ptc-lacZ* and (D and H) Ci-155 intensity profiles along the AP axis for single experiments ($n$ = 4 wing discs). (I) Diagram representing the structure of *gCi*, *Ci-1*, and *SV-1* transgenes, in which introns are deleted (boxed regions are exons with coding sequence in orange). In *SV-1*, the 3′-UTR was replaced by SV40 3′-UTR sequences (blue). (J–L) *ci* null wing discs with one copy of (J) *gCi*, (K) *Ci-1*, or (L) *SV-1*, stained for *ptc-lacZ* (red) and Ci-155 (white). (M) *ptc-lacZ* and (N) Ci-155 intensity profiles along the AP axis for single experiments ($n$ = 3 wing discs). (O–V) Wings from $ci^{Cell}/ci^{94}$ flies with one copy or two copies of (O and P) *gCi*, (Q and R) *gCi ATG-A*, (S and T) *gCi ATG-B*, (U) *Ci-1*, or (V) *SV-1*. AP, anterior/posterior.

*gCi WT*, while *SV-1* Ci-155 levels were only marginally lower (Figure 6, J'–L', and N). We were not able to collect enough rescued wing discs to measure *ci* RNA to determine if intron removal in *Ci-1* reduced Ci-155 protein because of reduced transcription, RNA stability, or translation. AP border expression of *ptc-lacZ* appeared normal in discs rescued by *SV-1* but was slightly reduced in the very few wing discs rescued by *Ci-1* (Figure 6, J–M).

Rescue of $ci^{Ce}/ci^{94}$ animals showed a similar pattern. *Ci-1* produced almost no adults, even when present in two copies, and did not support the normal v3–4 spacing endowed by two copies of *gCi WT* (Figure 6U and Table 2). *SV-1* rescued

**Table 2 Rescue to adulthood of $ci^{Cell}/ci^{94}$ flies by *gCi* transgenes**

| | *transgene/+*; $ci^{Ce}/ci^{94}$ (% *transgene/+*; $ci^{94}$/+) | *transgene/transgene*; $ci^{Ce}/ci^{94}$ (% *transgene/+*; $ci^{94}$/+) |
|---|---|---|
| *gCi* | 44 (*n* = 218) | 76 (*n* = 992) |
| *gCi ATG-B* | 20 (*n* = 208) | 92 (*n* = 286) |
| *gCi ATG-A* | 17 (*n* = 440) | 32 (*n* = 462) |
| *SV-1* | 0 (*n* = 192) | 21 (*n* = 843) |
| *Ci-1* | 0 (*n* = 546) | 0 (*n* = 941) |

no adults in one copy but in two copies rescue approached the efficiency of one copy of *gCi WT*, with a modest wing vein abnormality (Figure 6V and Table 2).

These results show that *ci* introns are important to support normal functional levels of Ci-155 and provide further evidence that relatively small reductions in Ci-155 levels compromise robust Hh signaling (measured by rescue efficiency and phenotypes) in a number of settings, including reduced *ci* gene dosage or the presence of constitutive Ci repressor.

The functional deficit of *Ci-1* relative to *SV-1* is likely due to reduced Ci-155 levels and *SV-1* undoubtedly has considerable ability to support Hh signaling. However, the surprisingly poor rescue of $ci^{Ce}$ animals by *SV-1*, given almost normal Ci-155 levels, suggests that intron removal may also compromise some regulatory input from Hh.

### *ci RNA as a direct target of Mago and Srp54 function in Hh signaling*

The significant, though incomplete, rescue activity of the *SV-1* transgene provided a way to test whether Mago or Srp54 acted directly on *ci* RNA splicing to impact Hh signaling. If so, we should see no effect of Mago or Srp54 inhibition on signaling through *SV-1*. We were able to test this most effectively in the context of *pka* mutant clones, where both *mago RNAi* and *Srp54 RNAi* reduced *ptc-lacZ* induction in otherwise wild-type wing discs (Figure 4, H–K, N, and O). When a single copy of the *gCi WT* transgene rescues a *ci* null, ectopic *ptc-lacZ* expression in *pka* mutant clones was much lower than in wild-type discs and not satisfactory for testing the effects of Mago and Srp54 inhibition (data not shown). However, *ptc-lacZ* expression could be increased to an intermediate level by using either a heterozygous *Su*(*fu*) background (Figure 7A) or two copies of the transgene (Figure 7K).

The level of *ptc-lacZ* induced in *pka* mutant clones in *Su*(*fu*)/+ flies with one copy of *gCi WT* (and otherwise *ci* null) was reduced ~25% by expression of *mago RNAi* (Figure 7, A, B, and I). *SV-1* supported a slightly lower level of *ptc-lacZ* induction than *gCi WT*, consistent with lower Ci-155 levels produced by *SV-1*, but *mago RNAi* produced no change (Figure 7, C, D, and I). Similarly, Ci-155 levels were reduced at the AP border of wing discs by *mago RNAi* for *gCi* but not *SV-1* (Figure 7, E–H, and J). These results are consistent with *mago RNAi* normally reducing Hh pathway activity by reducing the levels of the major spliced *ci* RNA (*ci-A*) and primary Ci-155 translation product.

The level of *ptc-lacZ* induced in *pka* mutant clones by two copies of *gCi WT* (and otherwise *ci* null) was reduced almost 20% by expression of *Srp54 RNAi* (Figure 7, K, L, and S). *SV-1* supported a lower level of *ptc-lacZ* induction and *Srp54 RNAi* produced no reduction in *ptc-lacZ* expression in the presence of only *SV-1* (Figure 7, M, N, and S). This result is consistent with *Srp54 RNAi* normally reducing Hh pathway activity in *pka* mutant clones by reducing the levels of spliced *ci* RNA.

*Srp54 RNAi*, in contrast to *mago RNAi*, inhibited Hh signaling in wild-type wing discs, suggesting that this action may not be mediated by reducing *ci* RNA levels (Figure 2, A–F). Indeed, we found that *Srp54 RNAi* still decreased *ptc-lacZ* expression at the AP border of wing discs expressing only *SV-1* (Figure 7, O–R and T). Elevated anterior Ci-155 in response to *Srp54 RNAi* was also evident in the presence of *SV-1*, confirming that this action of Srp54 is also not dependent on *ci* splicing (Figure 7, O'–R'). Thus, Srp54 affects *ci* RNA splicing slightly differently to Mago but with the same functional consequence of reducing *ci-A* RNA levels, leading to lowered rates of Ci-155 production and Hh pathway deficits in several settings, including *pka* mutant clones. Srp54 additionally, through currently uncharacterized mechanisms, appears to promote Ci-155 processing in anterior cells and Hh signaling at the AP border.

## Discussion

We used a genetic modifier screen to identify new components in Hh signal transduction. Surprisingly, the screen identified EJC components and an SR protein, all with established functions in regulating RNA. Further investigation revealed *ci* RNA as a direct target and clarified the roles of different *ci* RNAs and the levels of their primary translation products in Hh signaling (Figure 8).

### *Direct actions of Mago and Srp54 that impact Hh signaling*

Reducing the level of each of the core EJC components, Mago, Y14, and eIF4AIII, reduced Hh pathway activity under sensitized conditions, while inhibition of Btz did not affect Hh signaling. This pattern of EJC contributions has been observed previously for the archetypal studies showing EJC regulation of splicing of *MAPK* and *piwi* (Ashton-Beaucage *et al.* 2010; Roignant and Treisman 2010; Hayashi *et al.* 2014; Malone *et al.* 2014), and therefore suggested that RNA splicing may be the relevant EJC focus in Hh signaling. We found that loss of Mago reduced Hh pathway activity cell autonomously in *pka* mutant clones and in clones expressing activated Fu, indicating an effect on Hh signal transduction. Mago inhibition did not affect Hh signaling when mediated by a *ci* transgene (*SV-1*) that contained no introns, identifying *ci* RNA splicing as

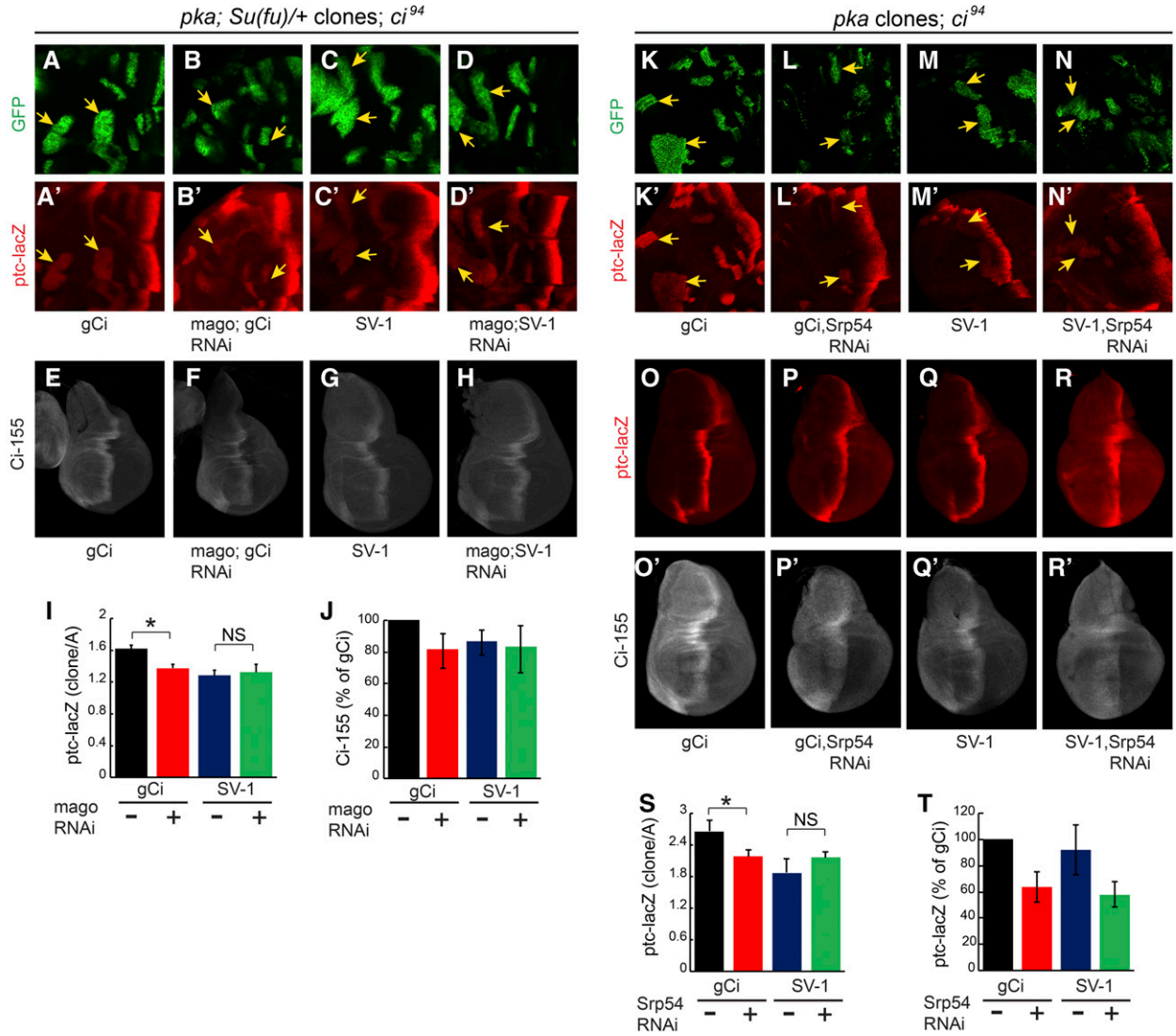


**Figure 7** An intronless *ci* transgene tests *ci* RNA splicing as a key target of Mago and Srp54. (A–D and K–N) Ectopic *ptc-lacZ* (red) induced in anterior *pka* clones (marked by GFP, green, arrows) in wing discs null for *ci* with (A–D) one copy of a *ci* transgene and heterozygous for *Su(fu)* or (K–N) two copies of a *ci* transgene, was measured in the presence or absence of (A–D) *mago RNAi* or (K–N) *Srp54 RNAi*. (A, C, K, and M) *ptc-lacZ* expression was higher for discs with *gCi* than for *SV-1* and was reduced by *mago RNAi* and *Srp54 RNAi* (A, B, K, and L) in the presence of *gCi* but (C, D, M, and N) not in the presence of *SV-1*. (I and S) *ptc-lacZ* expression in *pka* clones in discs of the designated genotypes relative to discs with *gCi* (and no RNAi), showing mean, SEM, and significant differences by Student's *t*-test (* $P < 0.05$) for (I) $n = 3$, $n = 3$, $n = 4$, and $n = 3$ clones, and (S) $n = 11$, $n = 9$, $n = 7$, and $n = 12$ clones in the order shown. (E–H) Ci-155 (white) in *ci* null wing discs with two copies of *gCi* or *SV-1* in the presence or absence of *mago RNAi* expression. (J) Maximal Ci-155 intensity as a percentage of wing discs with *gCi*, showing means and 95% C.I.s from two independent experiments of $n = 6$ discs for each condition. Ci-155 levels were reduced by Mago inhibition for *gCi* but not for *SV-1*. (O–R and T) *ptc-lacZ* (red) and Ci-155 (white) in *ci* null wing discs expressing *UAS-Diap1* with two copies of *gCi* or *SV-1* in the presence or absence of *Srp54 RNAi*. (T) Maximal *ptc-lacZ* intensity at the AP border as a percentage of wing discs with *gCi*, showing means and 95% C.I.s from two independent experiments of $n = 6$ discs for each condition. AP, anterior/posterior; RNAi, RNA interference.

the key Mago target. We also observed changes in the pattern of *ci* RNAs when Mago was inhibited. Specifically, the level of a minor alternatively spliced product (*ci-B*) was increased while the level of the major splice form (*ci-A*) was decreased by an amount commensurate with observed reductions in Ci-155 protein levels and Hh target gene inhibition (Figure 8).

The evidence for Srp54 acting directly on *ci* RNA splicing is similar to that for Mago in terms of cell autonomous action and a failure to influence Hh pathway activity when the only source of Ci protein is an RNA (from the *SV-1* transgene) that does not need to be spliced. However, unlike Mago depletion, inhibition of Srp54 reduced the levels of *ci* RNA measured across most introns but did not alter the ratio of *ci-A* and *ci-B* RNAs, so it is unlikely that Srp54 is acting on *ci* RNA in concert with EJC components, despite some evidence of association of Srp54 with EJCs in directing alternative splicing (Sakashita *et al.* 2004).

For Srp54, unlike Mago, we also found evidence for effects on the Hh pathway that are not mediated by alterations in *ci* RNA. First, Ci-155 protein was increased in anterior wing disc cells when Srp54 was inhibited (Figure 8). This most likely

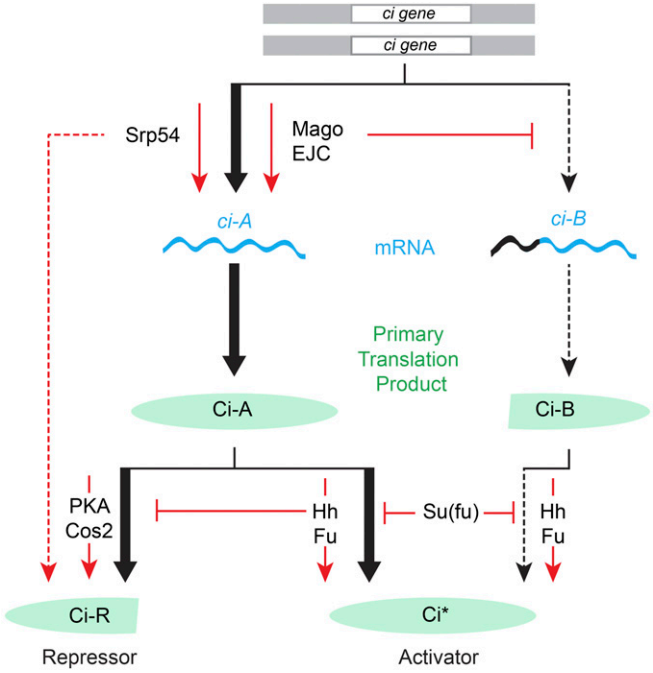


**Figure 8** Role of the EJC, Srp54, and *ci* RNA production in Hh signal transduction. In the absence of Hh, full-length Ci-155 protein is largely inactive and processed slowly, with the participation of PKA and Cos2, into active Ci-75 repressor. Hh signal transduction via activation of Smo leads to inhibition of Ci-155 processing, leading to a loss of Ci-75 repressor and a greater accumulation of Ci-155 primary translation product. Hh also promotes activation of Ci-155 via Fu kinase and in opposition to Su(fu). Activated Ci-155 is subject to Cul3-mediated degradation (data not shown). The role of Ci-155 levels in contributing to Hh target gene activation is not well-studied. Here, we have found that reduced rates of Ci-155 production due to reduced *ci* gene dose, genetic removal of *ci* intronic sequences, or reduced production of the major *ci* RNA (*ci-A*) through inhibition of core nuclear EJC factors or Srp54, reduce Hh pathway activity under conditions of submaximal activation (by Hh in the absence of Fu kinase, by synthetic Fu activation, or by loss of PKA or Cos2). Loss of Su(fu), like reduced Ci-155 production, only affects Hh pathway activity under an overlapping set of conditions for submaximal pathway activation, illustrating the potential for partial redundancy in regulating Ci-155 levels and Ci-155 activity. The minor *ci-B* RNA is increased in the absence of Mago and encodes a protein with normally regulated activator function but appears not to be processed to a functional repressor. EJC, exon junction complex; Fu, Fused; Hh, Hedgehog; PKA, Protein kinase A; Smo, Smoothened; Su(fu), Suppressor of fused.

reflects impaired Ci-155 proteolytic processing to Ci-75 repressor because Srp54 inhibition actually lowered Ci-155 levels when Ci-155 processing was eliminated in *pka* mutant clones. Second, Srp54 inhibition reduced *ptc-lacZ* induction at the AP border of normal wing discs, whereas twofold reduction in *ci* dosage or Mago inhibition had no effect. Moreover, AP border *ptc-lacZ* inhibition was not rescued by providing excess *ci* and it was still observed when the only source of *ci* was an intronless transgene. Thus, Srp54 has additional uncharacterized actions that impinge on Hh signaling.

### *Ci-155 protein levels in Hh signaling*

It has previously been recognized that Hh signaling alters both the amounts and activities of Ci proteins (Briscoe and Therond 2013). However, the impact of regulating Ci-155 levels has not yet been investigated carefully. In this study, we have altered the rate of production of the primary full-length Ci translation product through heterozygosity for *ci*, various *ci* transgenes, and Mago inhibition. We consistently observed that Hh pathway activity was altered by twofold or lesser changes in Ci-155 levels under conditions where normal Hh signaling was compromised (loss of Fu kinase) or only partially phenocopied (by loss of *pka* or synthetic Fu activation). The observed sensitivity to relatively small changes in Ci-155 makes it plausible that any mechanism that regulates Ci-155 levels, including regulation of *ci* splicing, contributes to normal Hh signaling (Figure 8).

### *Impact of alternative ci RNAs and ci splicing on Hh signal transduction*

Our observation that Mago inhibition altered the proportion of *ci-A* and *ci-B* RNA prompted investigation of potentially distinctive roles of these RNAs. Both public data and our investigation of wing disc RNA showed *ci-B* to be much less abundant than *ci-A*. Hence, we first considered the hypothesis that *ci-B* might encode a hyperactive form of Ci activator, potentially induced as a feed-forward mechanism to achieve the highest levels of Hh signaling. Our investigations did not support that speculation. A *ci* transgene lacking the initiator codon for Ci-B supported normal Hh signaling, while another transgene lacking the initiation codon for Ci-A, and therefore expected to produce more Ci-B protein than normal, was not hyperactive. Instead, the *gCi ATG-A* transgene product appeared to be a normally regulated activator that does not generate an active processed repressor. At present, we cannot therefore assign a distinct function to the N-terminally truncated protein that is predicted to be encoded by *ci-B* RNA.

We additionally tested the properties of two *ci* transgenes lacking any intronic sequences. *SV-1*, which included an *SV40* 3′-UTR in place of *ci* sequences, produced significantly more Ci-155 protein than *Ci-1* and had greater rescue activity in the absence of normal *ci* gene activity. Both were less active than a wild-type transgene but exhibited improved function in two doses. The dose-dependent and graded deficiencies of these transgenes provide further evidence of the importance of the levels of Ci-155 primary translation product for Hh signaling. We did not determine why intron removal reduced Ci-155 production, but two plausible possibilities are that the large first intron includes a transcriptional enhancer or that translation efficiency is compromised, as observed for some other genes following intron removal (Chorev and Carmel 2012). Although substantially reduced Ci-155 levels provide a sufficient explanation for major deficits of *Ci-1*, the rescue activity of *SV-1*, most notably in a $ci^{Ce}$ background, was lower than expected from measurements of Ci-155 levels. This quantitative limitation of the activity of an intronless transgene suggests that the presence of introns in *ci* may also serve a regulatory role that is important for *ci* to transduce Hh signals robustly.

When assessing the impact of a regulatory process in Hh signaling, it is important to consider the potential for partially or fully redundant mechanisms (Figure 8). Su(fu) provides a notable example of a factor that is central to the mechanism of Hh signal transduction in *Drosophila* and mammals, but only discernibly affects Hh pathway activity in *Drosophila* under conditions that perturb normal Hh signaling (Preat 1992; Zhou and Kalderon 2011). In fact, Mago affects Hh pathway activity under exactly the same conditions as Su(fu) (*e.g.*, in *pka* mutant clones or when Fu kinase is inactive) but not under normal conditions (Figure 8). Similarly, deficiencies of intronless *ci* transgenes are especially evident under sensitized conditions (reduced *ci* dosage, *pka* mutant clones, or in the presence of a constitutive repressor). To resolve the contribution of potentially regulated *ci* splicing, the EJC, Srp54, and translatable *ci* RNA levels to Hh signaling, it will be necessary to simultaneously eliminate regulation through Ci-155 activation, Ci-155 processing, and Ci-155 proteolysis individually, and perhaps in combination. At present, the studies described here have alerted us to the possibility that *ci* RNA transcription and processing, the EJC, and an SR protein splicing factor may all play a significant role in regulating the output of Hh signaling.

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# Chapter 4

## *Using Genomic Transgenes and the CRISPR Cas9 Gene Editing System to Understand How Ci Stability and Activity are Regulated by Hedgehog Signaling*

***Introduction***

Cubitus Interruptus (Ci) is the sole transcription factor of the Hedgehog (Hh) signaling pathway in flies; it is responsible for interpreting how much signal is present and relaying that information to the nucleus to determine which target genes are expressed. In order for Ci to perform these functions, multiple upstream signaling proteins dynamically regulate Ci processing, activation, and degradation (Briscoe and Therond 2013).

Previous research has used non-physiological conditions to investigate some, but not all, properties of Ci variants successfully. From biochemical binding and phosphorylation shifts together with the expression of UAS-Ci transgenes *in vitro*, we and others identified a series of phosphorylation sites that are necessary for Slimb binding and subsequent Ci-155 processing to Ci-75 repressor (Aza-Blanc, Ramirez-Weber et al. 1997) (Figure 1A). For example, 1) GST binding assays revealed that serine 849 was necessary for E3 ubiquitin ligase Slimb binding (Figure 1B), 2) *hh-lacZ* repressor assays in posterior wing disc cells determined that UAS-Ci-S849A cannot produce Ci-75 repressor (Figure 1I-J, and 3) *ptc-lacZ* activity assays showed that UAS-Ci-S849A activity was greater than that of an equivocally expressed UAS-Ci-Wt transgene (Smelkinson, Zhou et al. 2007) (Figure 1 C-H). The absence of processing was also evident by tagging UAS-Ci-S849A with a Myc tag in the C-terminus and monitoring full length Ci-155 levels, which were uniform throughout the wing disc, in contrast the to normal elevation of Ci-155 of the UAS Ci-Wt-Myc transgene product at the AP Border (Zhou and Kalderon 2010).

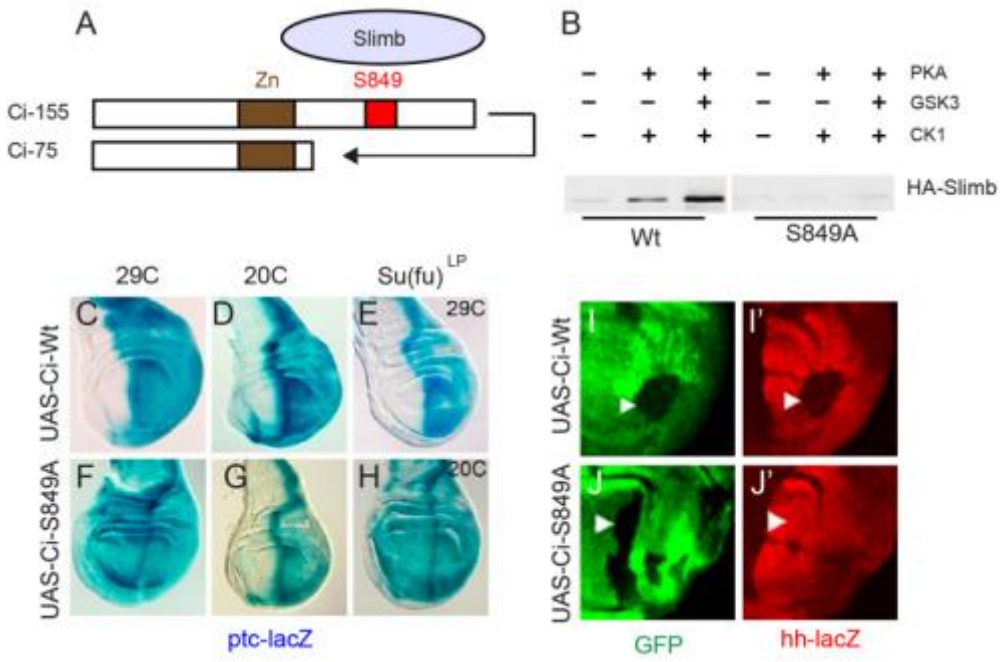



**Figure 1 UAS-S849A Blocks Slimb Binding and Subsequent Proteolysis:** adapted from (Smelkinson, Zhou et al. 2007) (A) Schematic of Ci-155 processing: Zn fingers are in brown and Phosphorylation target region by PKA, GSK3 and CK1 is in red. (B) GST binding assay looked at the binding of tagged HA-Slimb to UAS-Ci-Wt and UAS-Ci-S849A, mutation of Serine 849 to Alanine Blocks Slimb Binding (C-E) Staining for B-Galactosidase in wing discs expressing *ptc-lacZ*, UAS-Ci-Wt had ectopic expression of *ptc-lacZ* in the posterior but normal gradation at the AP border and absence of *ptc-lacZ* in the anterior with or without Su(fu). (F-H) UAS-Ci-S849A had ectopic anterior expression of *ptc-lacZ* when it was expressed at higher temperatures or in the absence of Su(fu). (I-I') The *hh-lacZ* assay indicates that in the absence of the GFP and *smo*, UAS-Ci-Wt promotes processing of Ci-75 repressor which then reduces the expression of *hh-lacZ*. (J-J') UAS-Ci-S849A does not reduce the *hh-lacZ* in the clone indicating that there is no repressor.

The Holmgren lab derived several P-element insertions of the Gal4 gene driven by Ci regulating sequences (*ci-Gal4*) that drove UAS transgene expression in a normal Ci pattern, it was restricted to the anterior of developing wing discs. They reported that *ci* null flies could be fully recovered by a combination of *ci-Gal4* and UAS-Ci-Wt transgenes. However, we tested this for several *ci-Gal4* and UAS-Ci insertions including those that were used successfully by the Holmgren lab at a variety of temperatures and observed virtually no rescue to adulthood (Figure 2 A-B) (Croker, Ziegenhorn et al. 2006). To assay the activity of Ci variants using UAS-Ci transgenes, it is essential that UAS-Ci-Wt can substitute for normal Ci activity. This has been tested in clones as well as in whole animals. We found that UAS-Ci expressed with C765-Gal4 did not restore anterior Engrailed expression or normal *ptc-lacZ* expression at the AP border in clones lacking endogenous Ci function. In fact, UAS-Ci expression alone had a dominate negative effect on Hh target gene expression (Figure 2C-D).

It is not clear why an optimal level of Ci expression could not be obtained with UAS-Ci transgenes. We considered whether the absence of introns may eliminate key regulatory input; further experiments with Ci splicing variants from Chapter 3 suggested that could be a contributing factor but is not the main reason for the lack of rescue. Regardless of underlying explanations, it is impractical to use UAS-Ci transgenes to measure normal activity.

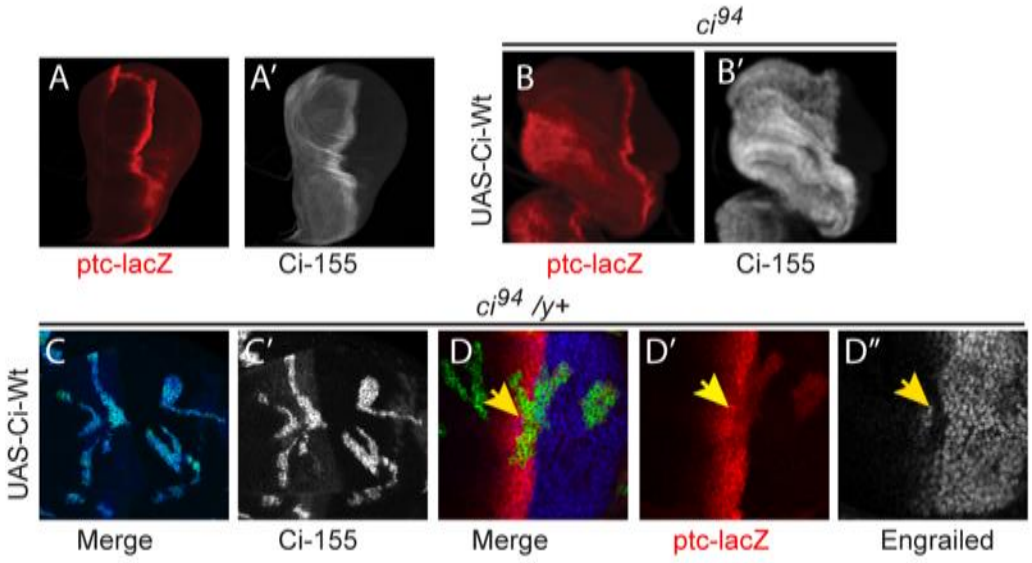



**Figure 2 UAS-Ci-Wt Cannot Rescue null ci94:** (A-A') The normal expression of *ptc-lacZ* and Ci-155 in a wild-type disc. (B-B') In a $ci^{94}$ null fly, UAS-Ci-Wt is being driven by *ci-Gal4* where *ptc-lacZ* and Ci-155 are restricted to the anterior but are highly over expressed. (C-C') Positively marked $ci^{94}$ clones coupled with the expression of UAS-Ci-Wt with a C765 Gal4 driver show elevated Ci-155 levels. (D-D") Positively marked $ci^{94}$ clones coupled with the expression of UAS-Ci-Wt show a dominative effect and reduce the expression of *ptc-lacZ* and Engrailed at the AP Border.

Therefore, we developed two strategies to study Ci expressed at physiological levels: genomic Ci (gCi) and CRISPR Ci (crCi). First, we devised a cloning strategy to create gCi by putting a 16kb genomic region of Ci that included upstream and downstream regulatory regions into an att site on the third chromosome (Figure 3C) (Methot and Basler 1999). A gCi-Wt transgene was readily able to rescue Ci null flies and behaved almost like a normal *ci* allele but with marginally lower *ci* expression and activity (Figure 3A). We created several gCi variants using this strategy.

Later we created a mutant *ci* allele without any artifact by using CRISPR in two rounds: in the first round, we put a mini-white gene in the first intron of *ci*, in the second round we selected against the mini-white gene and introduced our mutation of interest, replacing the DNA between the first intron and the 3'UTR. The CRISPR approach has the advantages of easier cloning steps and simpler construction of useful Drosophila stocks where the 3$^{rd}$ chromosome is readily manipulated (Figure 3D).

We are using gCi and crCi transgenes that encode processing resistant Ci-S849A to understand what happens when this mechanism is absent and to help to define any effects on Ci regarding activity from regulatory factors (Cos2 and PKA) that normally contribute also to processing. PKA phosphorylates Ci-155 at amino S838, S856, and S892 to promote a recognition site for both GSK3 and CK1 to further phosphorylate Ci-155 in a series of primed phosphorylations (Price and Kalderon 2002). This phosphorylation series eventually promotes Ci-155 processing to Ci-75 repressor by creating a binding site for Slimb that consists of the core peptide (844pSpTYYGpS849MQpS852) (Smelkinson, Zhou et al. 2007). We have used a physiologically expressed Ci-S849A transgene to study how Hh signaling regulates Ci activity and degradation in the absence of processing.

Previous studies have investigated Ci processing using physiologically expressed *ci* alleles from P element insertions/deletions, *ci-cell* and *ci-U*. *ci-cell* forms a constitutive Ci repressor that is truncated at amino acid 975; *ci-U* has a deletion between amino acids 611-760 and does not form Ci repressor (Methot and Basler 1999). Loss of PKA inhibits processing and stabilizes Ci-155 levels while ectopically increasing *ptc-lacZ* expression greater than loss of Slimb alone (Price and Kalderon 1999, Wang, Wang et al. 1999) (Ohlmeyer and Kalderon 1997) (Li, Ohlmeyer et al. 1995) (Jiang and Struhl 1995). This suggests that PKA can silence Ci-155

activity independent of processing but an alternative possibility was that Ci-155 processing was blocked more efficiently by loss of PKA than by loss of Slimb.

To discern between these two mechanism, UAS-Ci-U activity was studied in the wing disc where it was found to have increased activity by loss of PKA or by altering PKA sites 1-3 with UAS-Ci U and it was assumed that PKA silences Ci-155 activity through PKA target sites 1-3 (Wang, Wang et al. 1999). However, in subsequent studies using a Myc tag in the C-terminus, Ci-U was found to still undergo PKA dependent proteolysis, even though no Ci-75 repressor was found. Ci-S849A expressed at physiological levels is superior to UAS-Ci-U as a test this because it is immune to PKA directed proteolysis and its activity can be measured without artifact.

Costal2 (Cos2) acts as scaffold for kinases PKA, GSK3, and CK1 to promote Ci phosphorylation. Similar to PKA, it has been claimed Cos2 can inhibit Ci-155 activity independent of processing by binding to the Zn, CDN or CORD regions on Ci-155 (Jia, Amanai et al. 2002) (Price and Kalderon 2002) (Zhou and Kalderon 2010). However, the evidence for this hypothesis comes from greatly overexpressed Cos2 transgenes with clear dominant negative effects and the use of UAS-Ci transgenes. In one study, it was found that the activity of UAS-Ci-S849A in *smo* mutated posterior clones was increased by a loss of PKA or by loss of Cos2 in wing discs lacking Su(fu) (Marks and Kalderon 2011). Therefore, we wish to investigate Ci silencing via PKA phosphorylation and Cos2 binding using physiologically expressed Ci variants.

We are interested in determining how Su(fu) inhibits Ci-155. It is thought that Su(fu) mainly inhibits Ci activity by binding to a conserved site at the N-terminus of Ci-155, although a second weaker binding site has been identified on the C-terminus of Ci-155 (Han, Shi et al.

2015). The consequence of Su(fu) binding may be to limit the nuclear access of Ci-155 but the evidence for that is mixed; it is also possible that Su(fu) restrains Ci-155 activity in the nucleus by blocking a co-activator or by recruitment of co-repressors similar to Su(fu) inhibition on Gli proteins in mammals (Cheng and Bishop 2002, Zhang, Fu et al. 2013).

We are exploring the mechanism of Su(fu)/Ci inhibition further using Ci variants with altered Su(fu) binding motifs (SYGHI->SYAAD) or larger deletions regions that include this domain (Δ175-346, Δ230-272) to determine if SYAAD is sufficient for blocking Su(fu)/Ci inhibition or if a larger binding region is required. In addition to restricting Ci activity, Su(fu) is responsible for stabilizing Ci-155 and loss of Su(fu) results in Ci degradation in both anterior and AP border cells. Ci-155 is normally degraded in response to high levels of Hh signaling at the AP border where Su(fu) levels are reduced and Roadkill (Rdx) is transcriptionally induced by Hh signaling (Seong and Ishii, 2013).

Rdx, also referred to as HIB (Hh-induced Math and BTB domain-containing protein) and part of a Cul3 E3 ubiquitin ligase complex, recognizes and binds Ci-155 principally at six serine/threonine rich binding motifs (S1-S6) to promote its complete proteolysis. It has been postulated that Ci-155 degradation at the AP border is dependent not only on Hh induction of Rdx/HIB but is also restricted to nuclear, active Ci-155 and promoted by the hypothetical dissociation of Su(fu) from Ci-155 (Zhang, Shi et al. 2009) (Zhang, Zhang et al. 2006). It is unclear whether Su(fu) binding is an important determinant of Ci-155 proteolysis at the AP border and by what mechanism Ci-155 is degraded in anterior and AP Border cells in the absence of Su(fu). One possibility is that Su(fu) binds and blocks access of a proteolytic binding protein or because active Ci is inherently unstable and requires the binding to Su(fu) for stability. To test these possibilities, we wished to test Ci variants with reduced Su(fu) binding at the N-

terminus in the presence or absence of neighboring sequences and we will test the function of Ci molecules with Su(fu) binding sites in a different location. We also plan to test whether covalently attached Su(fu) with an altered Su(fu) binding domain can be rescued to test the idea that Su(fu)-Ci dissociation is a key regulation. Ci variants lacking Rdx binding regions will also be tested to understand better the role of direct action of Rdx on Ci -155 to regulate Ci-155 levels and activity the AP border.

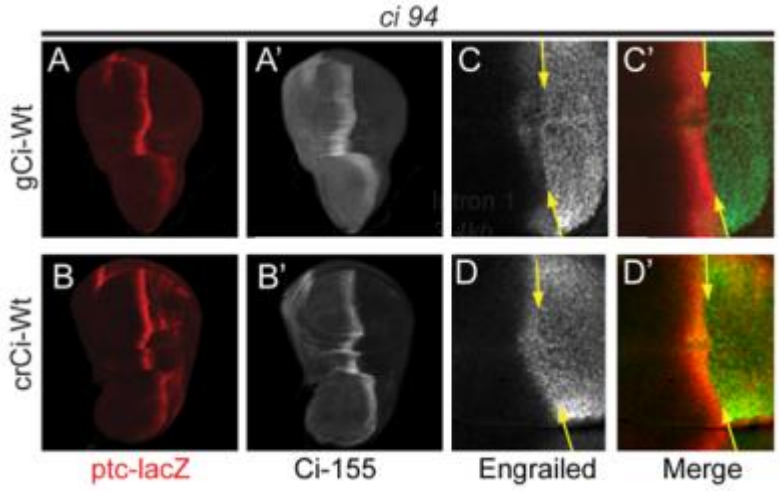
Lastly, we will investigate the mechanism of Ci activation by Fused kinase (Fu) (Zhang, Fu et al. 2013). We know that Fu is necessary for Ci activation but the critical targets of Fu are unknown. Fu must relieve inhibition by Su(fu) and Cos2 in order to activate Ci-155, so it was thought that Su(fu) and/or Cos2 were the direct phosphorylation targets. Su(fu) is phosphorylated in response to Hh at sites S321 and S324 by Fu but mutation of these sites does not affect Ci activity (Zhou and Kalderon 2011) (Ho, Suyama et al. 2005) (Oh, Kato et al. 2015). Fu also phosphorylates Cos2 at sites S572 and S973 and their phosphorylation events were claimed to promote Ci activation and stabilization on the basis of non-physiological transgenes (Ranieri, Ruel et al. 2012). When we altered these sites on a physiologically expressed Cos2, we found that they had no effect on Ci activity even when assayed in the presence of Su(fu) lacking Fu sites (Zadorozny, Little et al. 2015). Since Fu phosphorylation of Su(fu) and Cos2 does not promote Ci activation, we suggest that the crucial target site could be on Ci-155. We are currently testing this theory by investigating Ci variants lacking a variety of domains not required for inhibition by Su(fu) and by making point mutations within the Su(fu) binding region. If one of these alterations contains a critical Fu target site, then we would expect the Ci variant to not be responsive to Fu.

***Results***

*CRISPR and Genomic Transgenes Express Ci at Physiological levels and Rescue ci94*

Null Ci ($ci^{94}$) flies are embryonic lethal; gCi-Wt rescued $ci^{94}$ flies (gCi-Wt/Tm6b; $ci^{94}/ci^{94}$) to adulthood with only one copy of gCi-Wt. *ptc-lacZ* and Ci-155 staining were normal (comparable to Figure 2A) but in one copy of gCi-Wt, Engrailed was slightly weakened (Figure 3C). We also found that when we had one copy of endogenous Ci, one or two copies of gCi and one copy of $ci^{94}$ (gCiWt/Tm6B; $ci^{94}$/y+), the wing discs gave ectopic posterior *ptc-lacZ* which we believe is due to an artifact of one dose of the $ci^{94}$ allele but does not interfere with anterior signaling. We found gCi variants to be mostly reflective of endogenous Ci behavior albeit slightly weaker.

crCi-Wt flies were also able to rescue $ci^{94}$ flies to adult hood, they had normal *ptc-lacZ* and Ci-155 expression in the anterior (Figure 3B). Engrailed staining was better than one copy of gCi-Wt but there was a posterior *ptc-lacZ* artifact when there was one copy of $ci^{94}$ (Figure 3D). Since crCi "transgenes" are direct alterations of the *ci* gene, the effects of these Ci variants are highly unlikely to have any artifact. Therefore, both gCi and crCi transgenes are vastly more useful for studying Ci activity and stability than UAS-Ci.



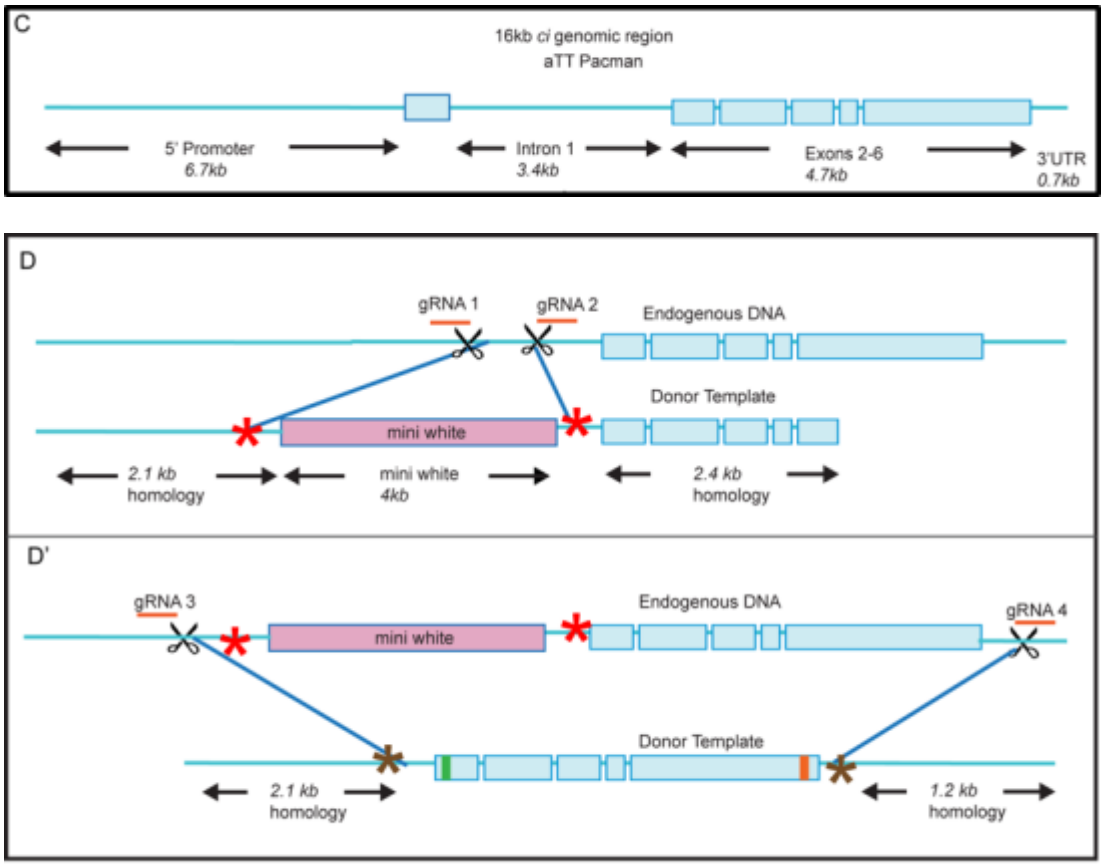



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**Figure 3 gCi WT and cr-Wt are able to rescue Ci:** (A-A') gCi-Wt has normal *ptc-lacZ* and Ci-155 levels, with (A"-A"') slightly weakened engrailed. (B-B") crCi-Wt is reflective of endogenous Ci and contains (B-B') normal *ptc-lacZ* in the anterior with some ectopic *ptc-lacZ* in the posterior, while (B"-B"') Engrailed staining is completely normal. (C) gCi is a 16kb genomic region of *ci* cloned into an ATT Pacman vector, it includes a 6.7kb upstream promoter region and 0.7kb of the 3'UTR. crCi was performed in two rounds: (D) the first round inserted a white plus gene into the first intron of endogenous Ci using two guide RNA's (orange) in the first intron and mutated PAM sites on the donor template (red star) (D') the second round replaced the entire coding region including the white plus gene with a donor template that contains an HA (orange) tag in exon 6 and a Flag (green) tag in exon 2 (mutant is marked by star) with a mutated

PAM sites approximately 30 basepairs outside the mutate PAM site for gRNA1 and in the 3'UTR 5kb away from gRNA 2.

*Ci Activity in the Absence of Processing*

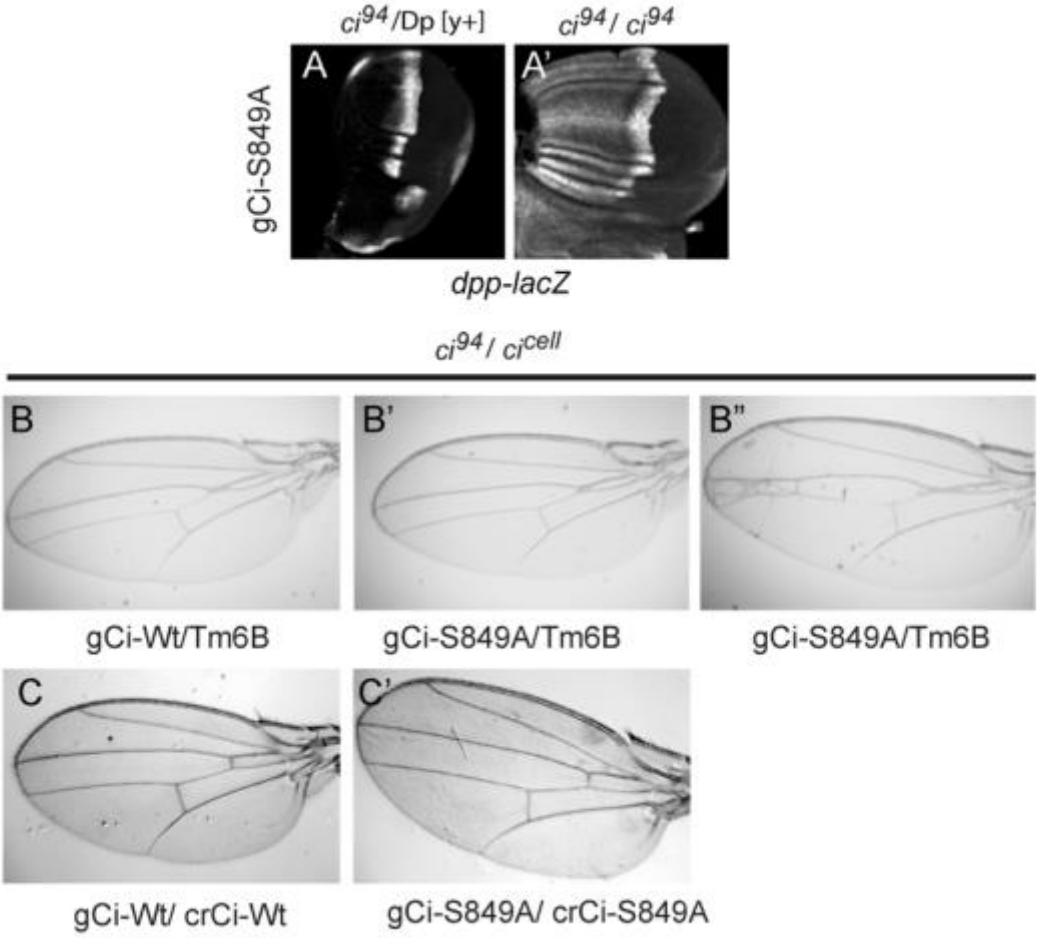



**Figure 4 Ci-S849A blocks processing with loss of Ci-75 Repressor function:** (A) *dpp-lacZ* staining is similar to wildtype when there is some Ci-75 present in Ci-S849A $ci^{94}$/Dp[y+] wing discs because Dp[y+] is a 4$^{th}$ chromosome marker that contains a wild-type copy of Ci, but (A') when Ci-S849A is the only source of Ci, the anterior region is expanded and *dpp-lacZ* is expressed uniformly throughout the anterior and higher at the AP border. When one copy of *ci*

$^{cell}$, a constitutive repressor, is introduced to (B) Ci-Wt, veins 3 and 4 are slightly pinched but this $ci^{cell}$ can rescue Ci-S849A flies to adult hood and have (B'-B") mostly normal wing vein patterning with variability. (C) gCi-S849A and crCi-S849 completely rescue the intervein spacing between veins L3 and L4 comparable to gCi-Wt and crCi-Wt.

We investigated whether Ci-S849A blocks processing and prevents the formation of Ci-75 as was suggested with the UAS-Ci-S849A transgene. If Ci-S849A lacked repressor function, then we would expect ectopic expression of *dpp* throughout the anterior and expansion of the anterior compartment. We looked at *dpp-lacZ* expression in third instar wing discs of S849A/Tm6B; $ci^{94}$ /Dp[y+], where $ci^{94}$ is over a chromosome marker (Dp[y+]). As expected, we found that there was not ectopic expression of *dpp-lacZ*, which is due to the presence of wild-type Ci from the Dp[y+] chromosome (Figure 4A). We then investigated *dpp-lacZ* expression in third instar wing discs of S849A/Tm6B; $ci^{94}/ci^{94}$ flies and found that they had ectopic expression of *dpp-lacZ* throughout the anterior of third instar wing discs (Figure 4B). Both gCi-S849A and cr-Ci-S849A were unable to permit the eclosion of adult flies, in the absence of any other Ci activity.

If the phenotype we see in Ci-S849A is due to a lack of repressor, then we would be able rescue Ci-S849A flies with the addition of $ci^{Cell}$, a *ci* allele that encodes a constitutive Ci repressor truncated at amino acid 975. We looked at gCi-Wt/Tm6B; $ci^{94}$ /$ci^{Cell}$ and *gCi*-S849A/Tm6B; $ci^{94}/ci^{Cell}$ flies and found that gCi-S849A flies were able to mature to adulthood. gCi-Wt had a consistent slight pinch between veins L3 and L4 due to the constitutive repressor and gCi-S849A had a variable phenotype, some adult wings looked similar to gCi-Wt and some adult wings were more pinched (Figure 4B). Next, we looked at whether increasing the dosage of

Ci could further rescue the L3-L4 fusion. We looked at gCi-Wt/Tm6B; crCi-Wt / $ci^{Cell}$ and gCi-S849A/Tm6B; crCi-S849A /$ci^{Cell}$ flies and found that both Ci-Wt and Ci-S849A in two copies fully rescued the intervein spacing between L3-L4. From these experiment, we found that Ci-S849A is an ideal transgene to study Ci activity and stability in the absence of processing and Ci-75 repressor is needed to keep *dpp-lacZ* off in anterior cells.

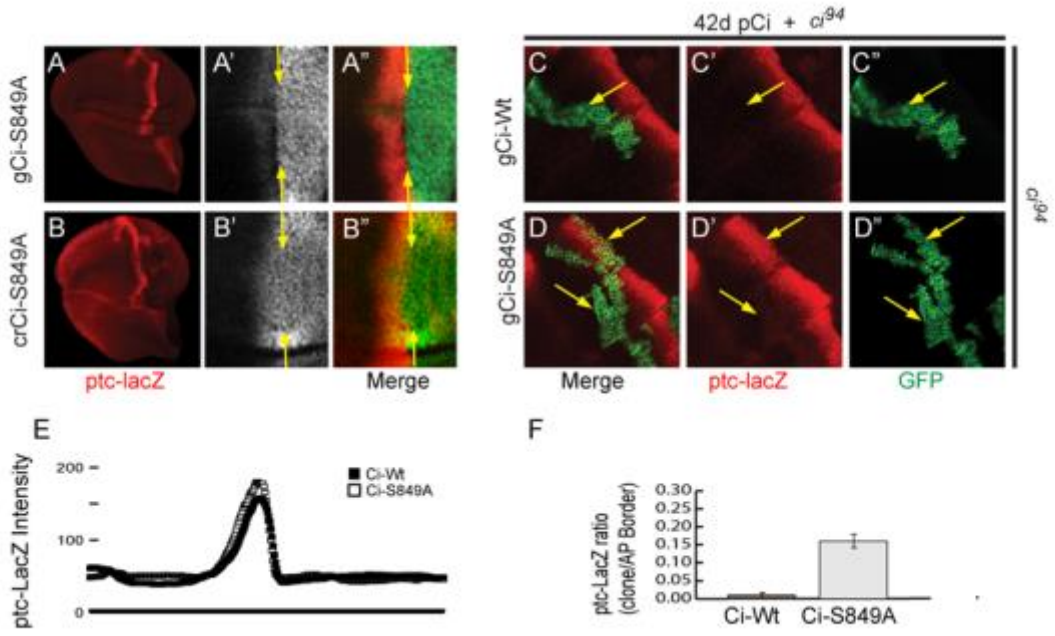


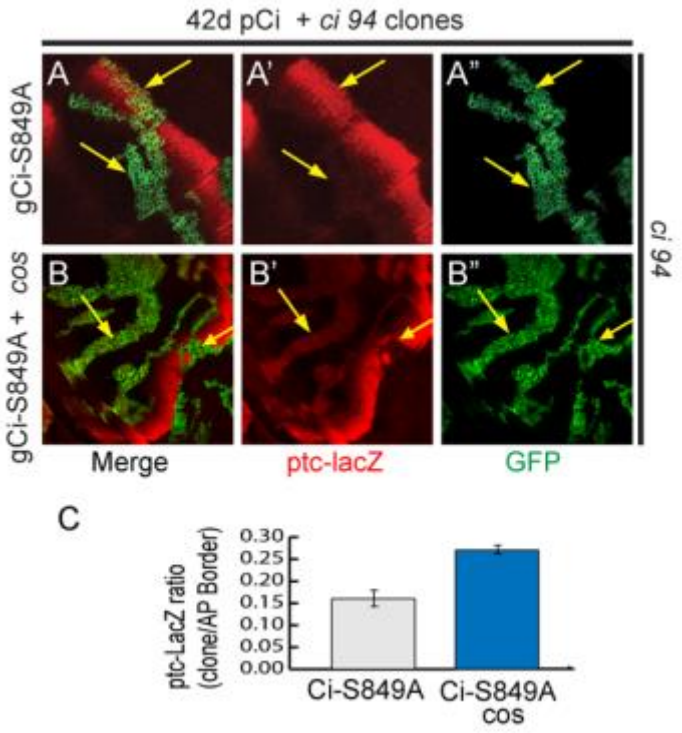

**Figure 5 Ci-S849A has normal graded signaling at the AP border and some ectopic activity in the Anterior:** In both (A-A") gCi-S849A and (B-B") crCi-S849A, wing disc anteriors were expanded but Hh signaling at the AP Border was normally graded for *ptc-lacZ* and Engrailed width was slightly greater than Wt. (C-D) We co-expressed wild-type 42dp[Ci] on the second chromosome with the genomic transgenes or the CRISPR alleles and induced $ci^{94}$ clones, so that in the clone we are only looking at the expression of the mutant transgenes to indicate if there is altered Ci activity and stability. I will refer to these type of clones as p[Ci]- $ci^{94}$ clones. In p[Ci]-

*ci*$^{94}$ clones, there was slightly elevated *ptc-lacZ* in (D-D") gCi-S849A discs but not in (C-C") gCi-Wt discs indicating ectopic activity.

Despite the absence of Ci repressor, Hh appears to regulate Ci-155 activity normally at the AP Border. This region, which is responsive to Hh, demonstrated graded *ptc-lacZ* induction in both gCi-S849A and crCi-S849A (Figure 5A-B); therefore, processing was not necessary for maintaining graded Ci activity. Engrailed and *ptc-lacZ* were expressed similarly in Ci-S849A and Ci-Wt at the AP Border, Ci-S849A had slightly higher *ptc-lacZ* when their profiles were measured and a slight expansion of Engrailed (Figure 5A'-A", 5B'-E"). We also looked at whether Ci-S849A induced any ectopic activity independent of the Hh signal by expressing a wild type copy of Ci (42dp[Ci]) on the second chromosome and gCi-S849A on the fourth chromosome throughout the fly made *ci*$^{94}$ clones which we call **p[Ci]-*ci*$^{94}$ clones**. We found that there was a small amount of increased *ptc-lacZ* in gCi-S849A (Figure 5D-D") but not gCi-Wt (Figure 5C-C", 5F). This indicates that when Ci is unable to be processed it still maintains its morphogenic activity gradient dependent on Hh and has some ectopic activity independent of Hh signaling.

*Cos2 and PKA effects on Ci Activity Independent of Processing*

Loss of PKA or Cos2 processing in the wing disc and induces ectopic Ci activity greater than the loss of *slimb* alone, so we hypothesized that PKA and Cos2 have an additional silencing effect on Ci activity. We tested this by 1) inducing *pka/cos2* clones in Ci-S849A wing discs and 2) creating Ci transgenes that block PKA phosphorylation or Cos2 binding.

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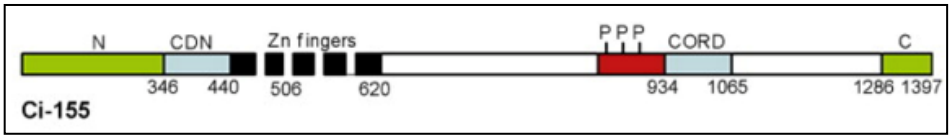


**Figure 6 Loss of Cos2 Increases Activity of Ci-S849A:** (A-C) In p[Ci]- *ci*$^{94}$ positively marked clones, the additional loss of Cos2 in gCi-S849A induced a greater amount of *ptc-lacZ* in the anterior and reduced *ptc-lacZ* at the AP Border compared to *ci94* clones alone. (D) Schematic of Ci-155 domains, Cos2 binds to the CDN, Zn Fingers and CORD regions.

First, we looked at Cos2's role in silencing by inducing p[Ci]- *ci*$^{94}$ clones with and without the additional loss of Cos2 (Figure 6B-B"). We compared the activity levels of *ci*$^{94}$ clones to *ci*$^{94}$ /*cos2* double mutant clones in gCi-S849 (Figure 6A-A"). The double mutant

*ci*$^{94}$/*cos2* clones expressed higher levels of *ptc-lacZ* than *ci*$^{94}$ clones, indicating that Cos2 has a role in Ci silencing independent of processing (Figure 6C). Since Cos2 binds to the CORD, CDN, and Zn finger regions on Ci we hypothesized that Cos2 can silence Ci-155 activity by binding to one of these regions. We deleted the CORD Region on Ci (gCi and crCi) and found that in both cases the flies were healthy, had comparable activity to wild type Ci. CiΔCORD also had elevated Ci-155 levels compared to Ci-Wt (Figure 7A-D).

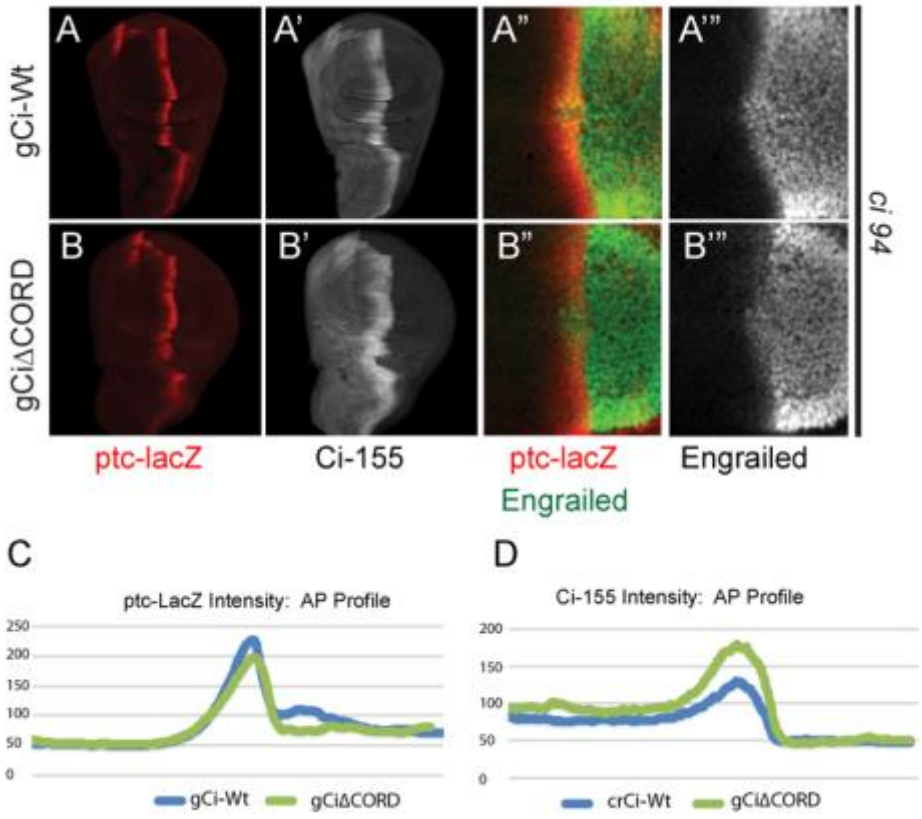


**Figure 7 CiΔCORD Has Slightly Lower Activity and Higher Ci-155 Levels: (**A-B) *ptc-lacZ* and (A"-B'") Engrailed expression levels in gCiΔCORD is comparable to gCi-Wt. (A'-B')) Ci-155 levels in Ci-ΔCORD were higher than Ci-Wt throughout the anterior. (C) Measurements of relative fluorescent intensity at the AP border showed that *ptc-lacZ* levels were slightly lower in

CiΔCORD but Ci-155 levels were elevated at the AP border and throughout the anterior in Ci-ΔCORD compared to Ci-Wt.

To determine if Cos2 silences Ci-155 activity by binding to the CORD region, we investigated whether CiΔCORD could increase *ptc-lacZ* in the absence of processing. Thus, we induced *pka* clones in both gCi-Wt (Figure 8A-A') and gCi-ΔCORD (Figure 8B-B') wing discs to eliminate processing as a potential variable and found that there was greater activity when we deleted the CORD region (Figure 7C). This suggests that Cos2 may negatively regulate Hh signal transduction by silencing Ci-155 via the CORD domain. We will further investigate this mechanism by using our crCi-S849A and crS849AΔCORD flies and inducing *cos2* clones. If Cos silences Ci-155 through the CORD domain then we would expect there to be ectopic *ptc-lacZ* in *cos2* clones in Ci-S849A compared to the rest of the disc; in CiS849AΔCORD, we would expect there to be higher levels of *ptc-lacZ* throughout the disc but no discernible increase in the cos2 clone (yet it would be the same intensity of the *cos2* clone in S849A).

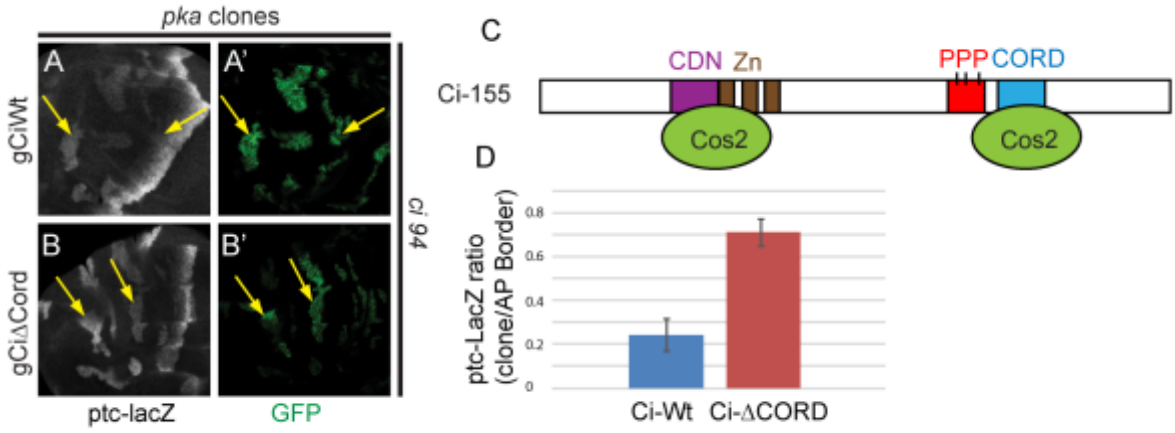

**Figure 8 Ci-ΔCORD has Higher Activity in PKA clones:** *pka* clones in (A) gCi-Wt and (B) gCi-ΔCORD have ectopic expression of *ptc-lacZ*. (C) Graphical representation of Cos2 Binding Ci-155. (D) By taking the ratio of the anterior clone to the AP Border, we found ectopic *ptc-lacZ* was greater in Ci-ΔCORD than Ci-Wt.

To investigate whether PKA also had a role in silencing Ci-155 activity, we induced *pka* clones in gCi-Wt (Figure 9A-A") and gCi-S849A (Figure 9B-B") discs. We found that *ptc-lacZ* was induced at higher levels in *pka* clones for gCi-Wt but not for Ci-S849A. However, the elevated *ptc-lacZ* for *pka* clones in gCi-Wt is notably weaker than two copies of endogenous Ci; one explanation for weakened expression of *pka* clones in Ci-S849A could be due to the single copy of the transgene. Therefore, we additionally took away a dose of Su(fu) to enhance activity in both gCi-Wt (Figure 9C-C") and (Figure 9D-D") gCi-S849A. Despite a higher increase in *ptc-lacZ* for gCi-Wt, loss of *pka* did not induce ectopic activity in the gCi-S849A transgene. We suspect that the Ci-S849A transgene is an unsuitable test for PKA silencing because it has muted activity and therefore we cannot readily confirm whether PKA has an additional role in Ci-155 silencing.

We will continue to test PKA silencing Ci-155 activity by using crCi transgenes that we created which have mutant PKA phosphorylation sites. We have mutated the first three sites on crCi transgene called crCiP(1-3)A and we will induce *pka* clones. If there is ectopic *ptc-lacZ* in the *pka* clones then it indicates that PKA can possibly silence Ci-155 by phosphorylating PKA target sites 4-5. We will then confirm this by creating *pka* clones in crCiP(1-5)A; if these sites are necessary for Ci silencing then we would not expect higher induction of *ptc-lacZ* in *pka* clones. Furthermore, we can use an activated PKA UAS-mC* transgene to overexpress PKA and

determine if processing resistant transgenes such as Ci-S849A and Ci(P1-3A) are further silenced by overexpressed PKA.

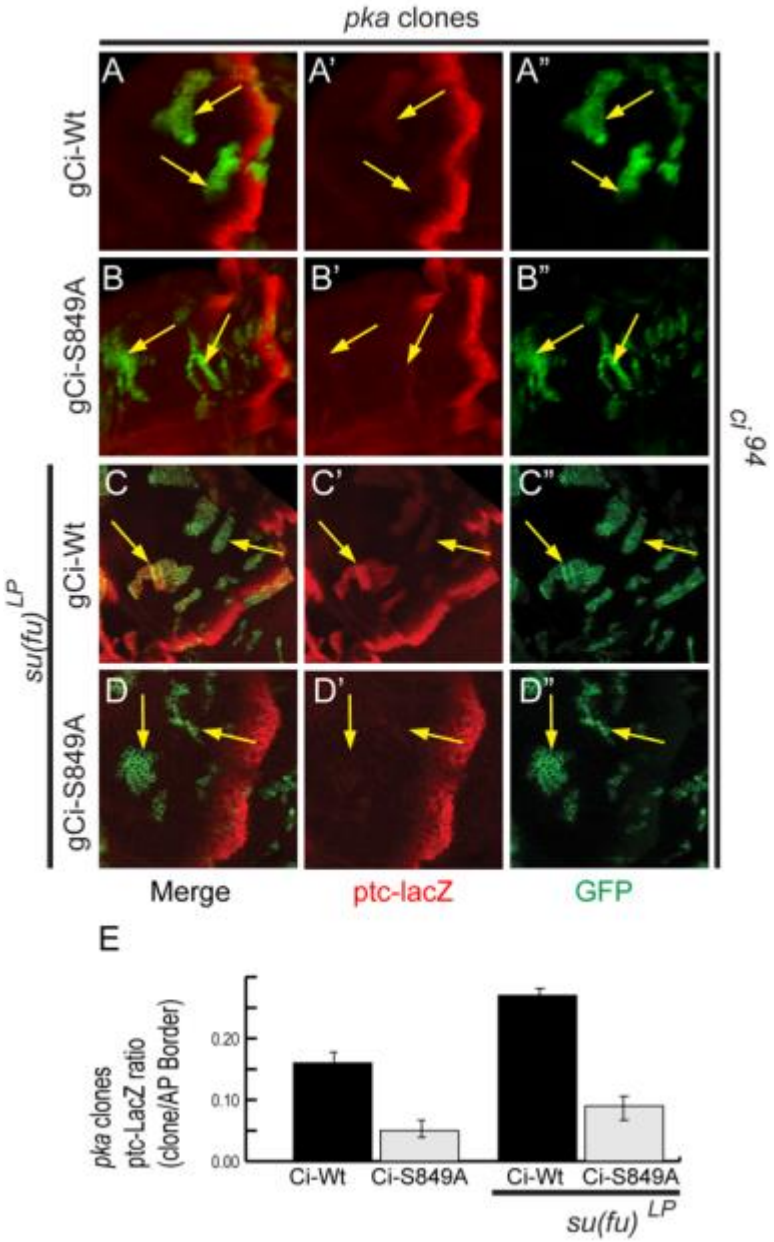


**Figure 9 *pka* Clones Do Not Induce Ectopic *ptc-lacZ* in Ci-S849A:** (A-A") Positively marked *pka* clones induced moderate *ptc-lacZ* in one copy of gCi-Wt. (B-B") There is no additional ectopic *ptc-lacZ* when looking at *pka* clones in Ci-S849A compared to the rest of the anterior. (C-C") Loss of Su(fu) increased the amount of ectopic *ptc-lacZ* in *pka* clones for gCi-Wt. (D-D")

*pka* clones in gCi-S849A had minimal induction of *ptc-lacZ* compared to the rest of disc even in the loss of Su(fu) (E) Overall measurements of the clones were analyzed to show that loss of *pka* induced less ectopic *ptc-lacZ* in gCi-S849A compared to Ci-Wt.

*Contributions of Fused Kinase on Ci Activity Independent of Processing*

Fu is necessary for Ci activation and loss of Fu kinase activity causes a loss *ptc-lacZ* at the AP Border. We show that when there is no Fused activity in Ci-S849A (Figure 10B), *ptc-lacZ* looks comparable to Ci-Wt (Figure 10A) but there is still a slightly expanded disc. This suggests that Fu is still necessary for Ci-155 activity independent of processing. We further investigated the role on Ci activity, by inducing UAS-Gap-Fu clones in gCi-Wt and gCi-S849A wing discs. UAS-Gap-Fu has palmitoylation signal attached to the N-terminus of Fu which localizes Fu at the membrane and induces ectopic Fu activity (Claret, Sanial et al. 2007). In UAS-GapFu clones, we found that there was a weak induction of *ptc-lacZ* levels in gCi-Wt discs (Figure 10A-'A'") but extremely high levels of *ptc-lacZ* in gCi-S849A wing discs (Figure 10B'-B'"). Furthermore, Ci activity in the Gap-fu clones for Ci-S849A was comparable to Hh signaling at the AP border (Figure 10C).

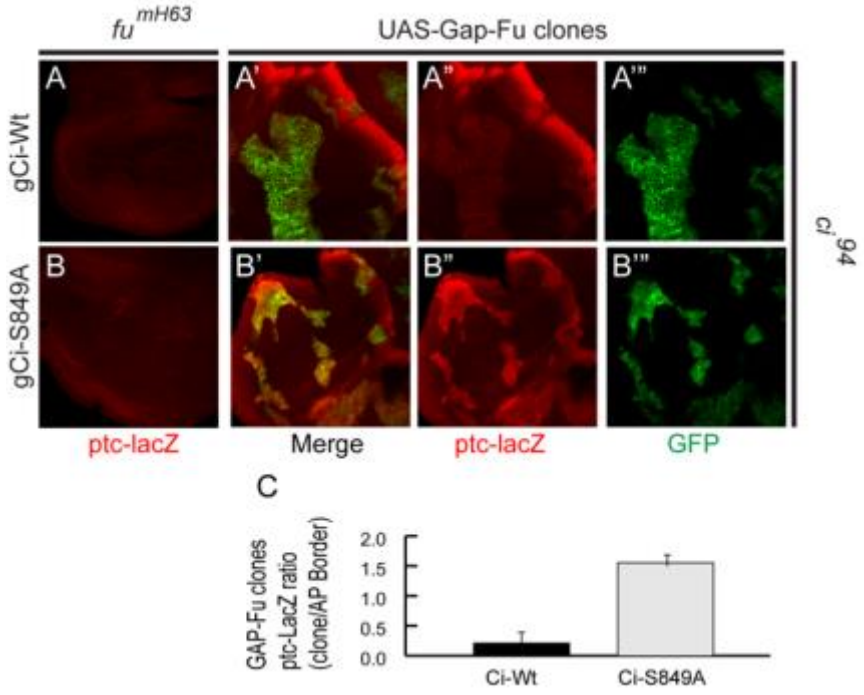


**Figure 10 The Role of Fused Kinase Independent of Processing** (A,B) Mutant *fu* reduces *ptc-lacZ* staining at the AP Border in both gCi-Wt and gCi-S849A. Activated Fused clones moderately increase *ptc-lacZ* in (A'-A"') Ci-Wt but greatly increased *ptc-lacZ* in (B'-B"') gCi-S849A. (C) The ratio of the clone intensity to the AP Border was measured and found to be significantly greater in gCi-S849A.

*Ci Stabilization in the Absence of Processing*

We investigated how Ci-155 levels are affected in the absence of processing. As expected, lack of processing in the gCi-S849A transgene had elevated Ci-155 levels throughout the anterior of the wing disc compared to Ci-Wt (Figure 11A, B). At the AP border, Ci-155 levels are reduced in Ci-Wt and have a sharper decline in Ci-S849A. Ci-degradation at the AP border is due to Hh regulated expression of Roadkill (Rdx)/Hib, a protein that binds Ci at multiple sites to promote its complete proteolysis.The combination of gCi-S849A and a

regulated copy of 42dp [Ci-Wt] continued to have increased Ci-155 levels throughout the disc but did not have an expanded anterior due to some repressor being present from the regulated 42dp [Ci-Wt] (Figure 11B'").

We will continue to investigate Rdx mediated proteolysis to determine if protein levels are important for activity, by using Ci variants that have altered Rdx binding sites: Ci-RdxS3-5 and Ci-S849A-S3-5. If these binding are sufficient to block Rdx binding to Ci-155, we would expect these variants to rescue the Ci proteolysis at the AP border. Furthermore, we will knockdown Rdx with RNAi in the wing disc of Ci-Wt, Ci-RdkS3-5 and Ci-S849A-S3-5 to confirm that they fully block Rdx to Ci-155 binding. If Ci-RdkS3-5 and Ci-S849A-S3-5 do not completely rescue the Ci-155 levels at the AP border or Rdx RNAi has a greater effect on Ci-155 stability than the transgenes, we will alter Rdx 1,2, and 6 motifs which were found to be less critical than 3-5 in *in vitro* studies. We will also investigate Ci-RdkS3-5 and Ci-S849A-S3-5 with and without Su(fu) to determine if Su(fu)-modulated proteolysis also contributes to Ci degradation at the AP border.

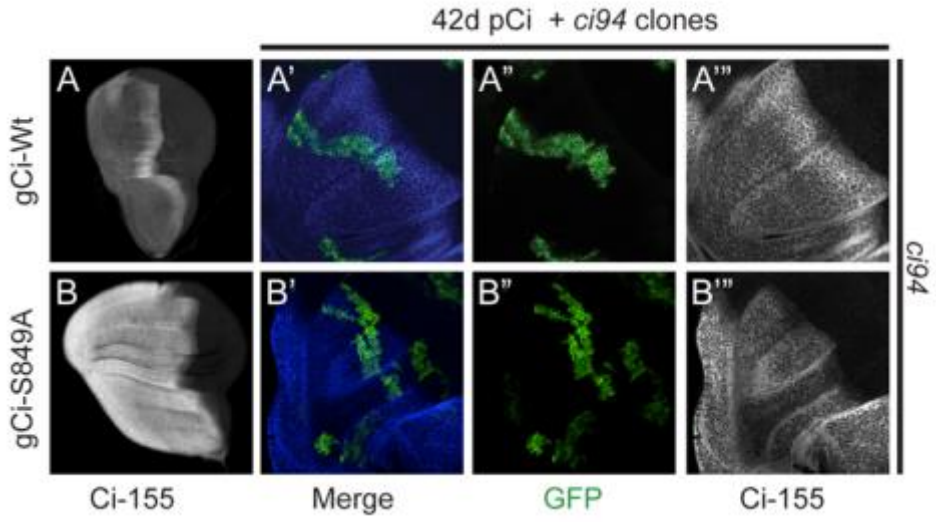


**Figure 11 Ci-S849A stabilizes Ci-155 levels Throughout the Anterior:** (A) In gCi-Wt whole disc, there is a normal Ci-155 profile, low in the far anterior, high at the AP border and slight

degradation near the posterior. (B) In gCi-S849A Ci-155 levels are high in the far anterior continuing to the AP border and then a sharp decline closer to the posterior. (A'-B'") In p[Ci]-*ci*$^{94}$ clones, Ci-155 level are unchanged for gCi-Wt and gCi-S849A.

Gap-Fu, Cos2, and PKA effects on Ci Stability Independent of Processing

We investigated to what extent Cos2 and PKA contribute to Ci-levels independent of processing. We found that *pka* clones maintained Ci-155 levels identical to gCi-S849A alone, confirming that S849A is insensitive to PKA dependent processing (Figure 12A-B). To investigate whether Cos2 contributed to Ci-155 stability in the absence of processing, we induced p[Ci]- *ci*$^{94}$ *cos2* clones in gCiWt and gCi-S849A (Figure E-F). We found that loss of Cos2 in gCi-Wt stabilized Ci-155 levels due to its role in processing, but *cos2* clones in gCi-S849A destabilized Ci-155 levels compared to the rest of the disc. This suggests that Cos2 may have an additional role to stabilize full length Ci-155 or higher expression of Rdx due to ectopic activity. We examined how Gap-Fu would affect Ci-155 levels in Ci-S849A. Activated Fu greatly increased Ci activity in gCi-S849A, but significantly decreased Ci-155 levels in Ci-S849A (12C-D) We presume that this is similar to the degradation mechanism at the AP Border.

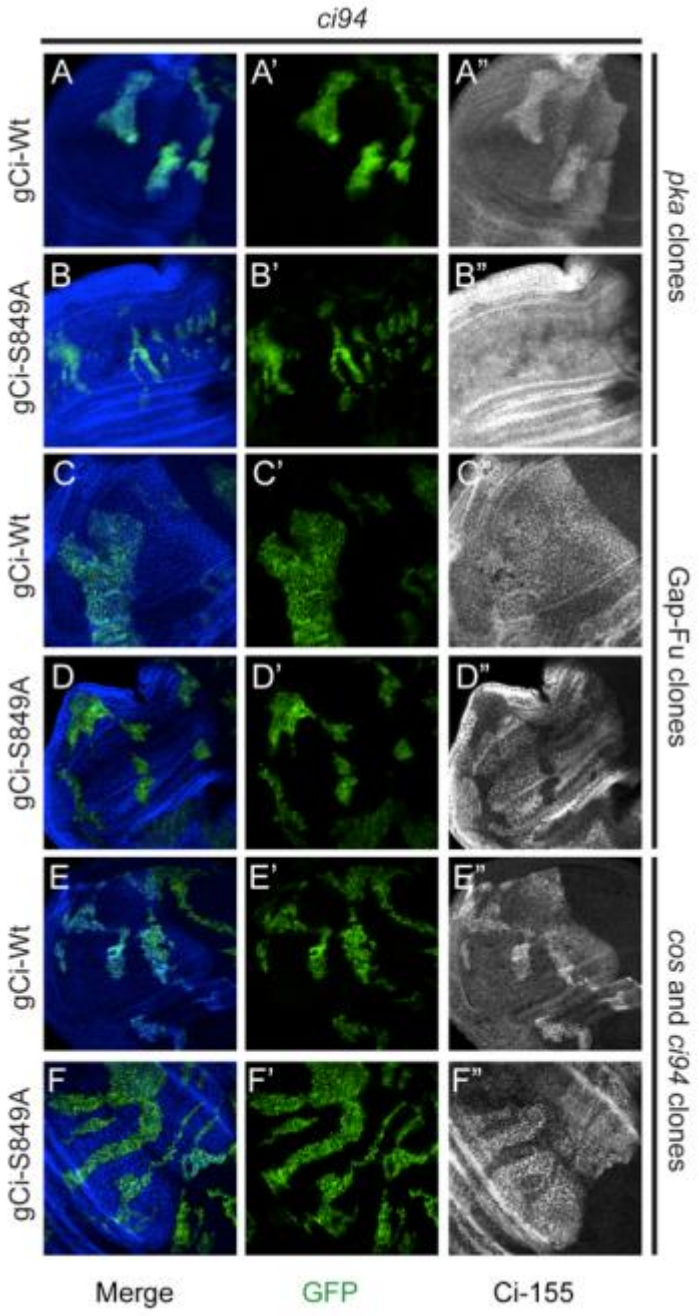


**Figure 12 Ci-155 is Destabilized in Gap-Fu and Cos2 but not PKA clones Independent of Processing:** (A-A") *pka* clones stabilize Ci-155 levels in gCi-Wt. (B-B") In gCi-S849A wing discs, Ci-155 are already stabilized due to loss of processing and loss of *pka* has no additional effect. UAS-Gap-Fu clones moderately stabilize Ci-155 levels in (C-C") gCi-Wt discs but

drastically destabilize Ci-155 levels in (D-D") gCi-S849A discs due to the increase in activity. In p[Ci]- *ci*$^{94}$ *cos2* clones stabilize Ci-155 levels in (E-E") gCi-Wt discs due to processing but in (F-F") gCi-S849A discs, Ci-155 is less stable.

*How does* Su(fu) *Inhibit Ci-155 activity?*

Su(fu) supposedly binds to Ci-155 at an N-terminus region and C-terminus region to stabilize Ci-155 levels and inhibit Ci-155 activity, but the mechanism of inhibition is not well understood. To investigate this mechanism, we have mutated the N-terminus binding site, SYGHI to SYAAD, on gCi and crCi to determine if Ci-SYAAD could replicate the loss of Su(fu) phenotype. We measured Ci-155 destabilization caused by the loss of Su(fu) and compared Ci-155 and *ptc-lacZ* profiles to a wild type wing disc (Figure 13A-B). We then investigated whether Ci-SYAAD caused the same amount of Ci-155 destabilization. We found that Ci-SYAAD had Ci-155 reduction similar to loss of Su(fu) alone (Figure 13C-D), which indicates that Ci-SYAAD has impaired binding to Su(fu).

To investigate whether Ci-SYAAD completely blocks Su(fu) binding, we examined whether Ci-SYAAD could rescue *fu*$^{mH63}$*: Su(fu)*$^{LP}$ /+ flies. *fu*$^{mH63}$*: Su(fu)*$^{LP}$ /gCi-Wt*; ci*$^{9}$ /*ci*$^{94}$ has greatly reduced *ptc-lacZ* expression at the AP border due to the loss of Fu kinase activity and only one copy of Ci-Wt (Figure 14A). *fu*$^{mH63}$*: Su(fu)*$^{LP}$ /gCi-SYAAD*; ci*$^{9}$ /*ci*$^{94}$ rescued *ptc-lacZ* expression at the AP border but only partially (Figure 14B. *fu*$^{mH63}$*: Su(fu)*$^{LP}$ *: Su(fu)*$^{LP}$ */;* crCi-Wt /*ci*$^{94}$ *and fu*$^{mH63}$*: Su(fu)*$^{LP}$ *: Su(fu)*$^{LP}$ */;* crCi-SYAAD /*ci*$^{94}$ rescued *ptc-lacZ* expression greater than *fu*$^{mH63}$*: Su(fu)*$^{LP}$ /gCi-SYAAD*; ci*$^{9}$ /*ci*$^{94}$ (Figure 14C-D). This data suggests that that Ci-SYAAD lacks Su(fu) binding but does not include the entire Su(fu) binding region.

We hypothesize that a larger deletion that includes SYGHI is required for complete Su(fu) binding so we are investigating Ci-transgenes Δ230-272 and Δ175-346. Ci-Δ230-272 and Ci-Δ175-346 have reduced Ci-155 levels comparable to SYAAD. In terms of Ci activity, *ptc-lacZ* was unchanged for loss of Su(fu), SYAAD, Δ230-272, and Δ175-346 but there was variable expansion for the wing discs in SYAAD as well as complete expansion for wing discs in Δ230-272 and Δ175-346 (Figure 13C-K). This is different from a $Su(fu)^{LP}$ mutant which has normal sized discs and readily reaches adulthood. Expansion of the wing disc is most likely due to ectopic expression of *dpp* but despite this defect, Ci-SYAAD is able to occasionally mature to adult hood. Adult Ci-SYAAD flies can have normal looking wings or have intervein expansion between veins 3 and 4, while Δ230-272 and Δ175-346 do not make it to adulthood and obtaining third instar larvae is not efficient. In conclusion, Ci-SYAAD, CiΔ230-272, and Ci-Δ175-346 transgenes have destabilized Ci-155 comparable to the *Su(fu)* mutant but have additional effects on activity most likely due to defects in the Ci-75 repressor activity. We will investigate this by examining Ci-SYAAD, CiΔ230-272, and Ci-Δ175-346 co-expressed with $ci^{cell}$ to see if this can rescue the expanded anterior.

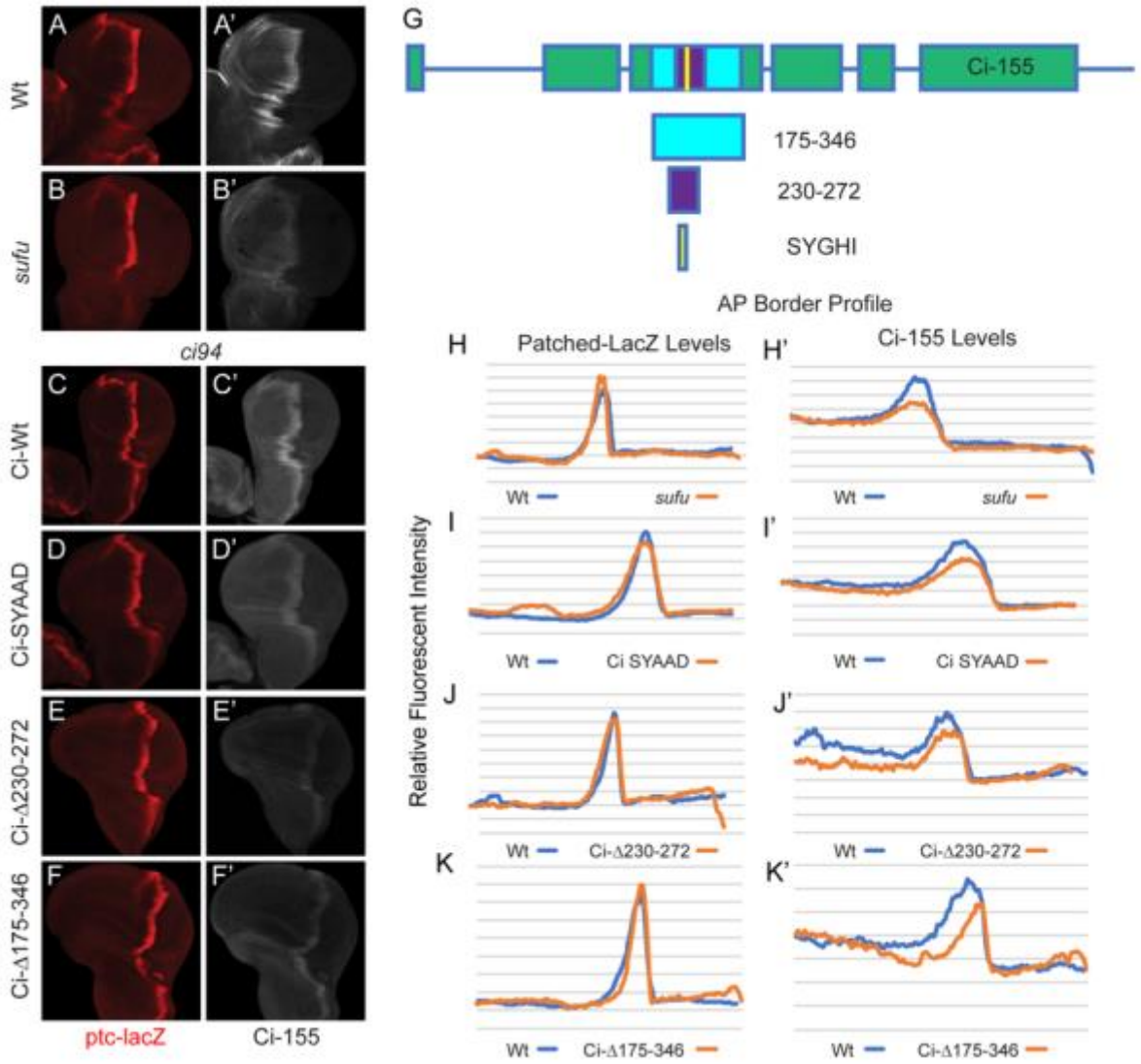


**Figure 13 Ci-SYAAD, Δ230-272, and Δ175-346 Reduce Ci-155 Stability Similar to Loss of *Su(fu)$^{LP}$* and have additional effects on repressor activity:** (A,B) *ptc-lacZ* levels were comparable between *Su(fu)$^{LP}$* and Wt wing discs but (A',B') *Su(fu)$^{LP}$* had decreased of Ci-155 levels, which was measured across the AP Border (H-H') . (D-D', I-I') gCi-SYAAD had *ptc-lacZ* levels similar to gCi-Wt and lower Ci-155 levels as well as a variably expanded anterior. (E-E', J-J') gCi-Δ230-272 had *ptc-lacZ* levels similar to gCi-Wt and lower Ci-155 levels as well

as an expanded anterior. (F-F', K-K') gCi-Δ175-346 had *ptc-lacZ* levels similar to gCi-Wt and lower Ci-155 levels as well as an expanded anterior.

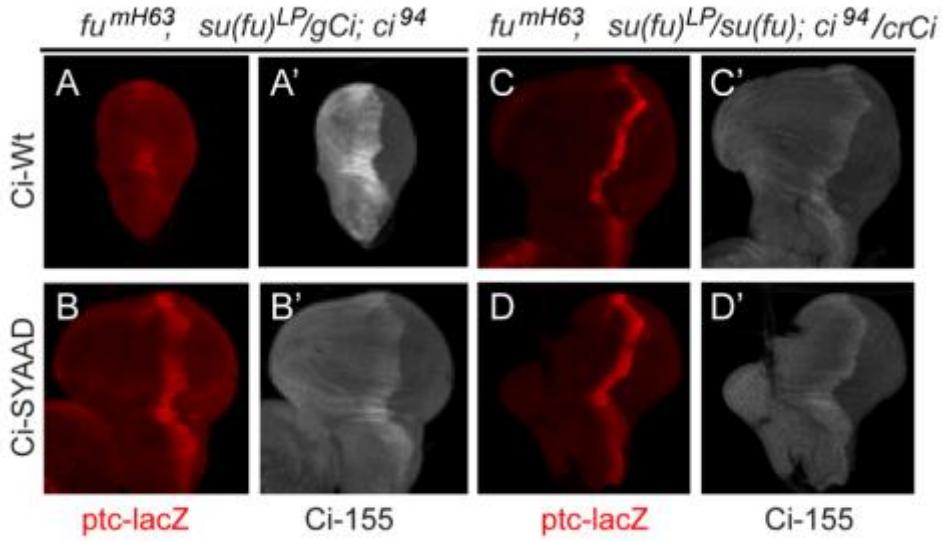


**Figure 14 Ci-SYAAD Partially Rescues Fused mutation:** (A-A') In *fu$^{mH63}$; Su(fu)$^{LP}$*/gCi-Wt; *ci94* flies, *ptc-lacZ* staining is greatly reduced and Ci-155 is stabilized in gCi-Wt wing discs. (B-B') *ptc-lacZ* staining is increased and Ci-155 is decreased in gCi-SYAAD wing discs. (C-C') *ptc-lacZ* staining was increased in *fu$^{mH63}$; Su(fu)$^{LP}$/Su(fu)$^{LP}$; crCi-Wt/ci$^{94}$* wing discs and was similar for (D-D') *fu$^{mH63}$; Su(fu)$^{LP}$/Su(fu)$^{LP}$; crCi-SYAAD/ci$^{94}$* wing discs while Ci-155 levels were lower than wild type for both crCi-Wt and crCi-SYAAD.

*Mechanism of Ci Activation Through the Actions of Fused Kinase*

We are investigating the mechanism of Ci activation by examining whether Ci-155 is a direct target for Fu phosphorylation. We suspect that if Ci is a direct target, the key phosphorylation site could be near the Su(fu) binding region because Su(fu) inhibits Fu kinase

activity. Therefore, we are investigating various point mutations and deletions within the Δ175-346 region, and examining whether they are responsive to activated Fu, if they are not then they are likely to be a critical Fu target site.

We induced p[Ci]- $ci^{94}$ clones in SYAAD, Δ230-272, and Δ175-346 with and without activated Fu. Ci-SYAAD, did not have ectopic activation alone but was very responsive to activated Fu (Figure 15 A-C). CiΔ230-272 had very minimal ectopic *ptc-lacZ* on its own (Figure 16A-C) and was also responsive to activated Fu but not to the same extent as SYAAD suggesting that there might be some target sites in that region. CiΔ175-346 had ectopic expression of *ptc-lacZ* on its own and comparable levels when co-expressed with activated Fu (Figure *17A*-C). We propose that there may be critical Fu targets sites within this region, but the low activity might be due to the lower Ci-155 levels. We will continue to investigate Ci as a target of Fu phosphorylation by using Ci variants Δ175-230, Δ272-346, and we will further examine CiΔ230-272 by creating point mutations on potential phosphorylation sites in this region.

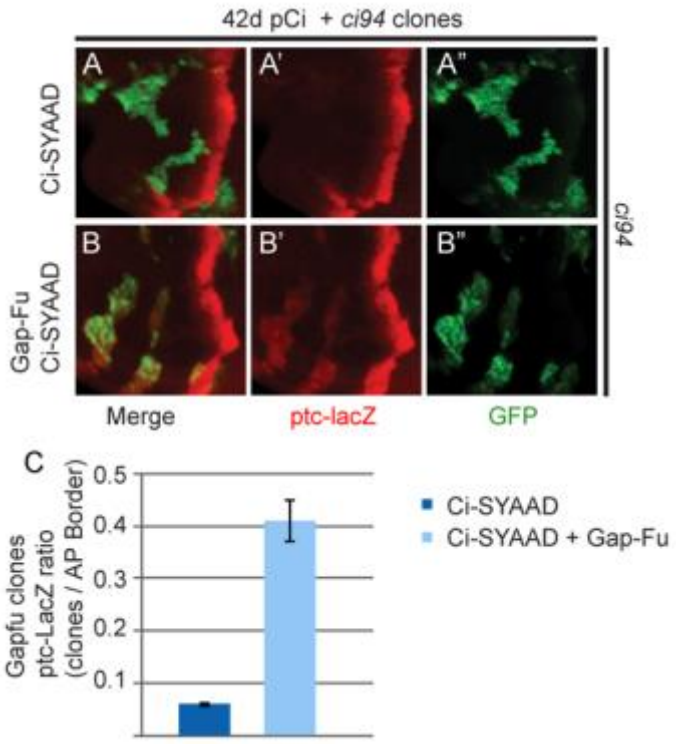


**Figure 15 gCi-SYAAD Does Not Have Ectopic Activity and Is Responsive to Activated Fused:** (A-A") In positively marked p[Ci]- *ci*$^{94}$ clones where gCi-SYAAD expressed throughout the wing disc, gCi-SYAAD did not induce ectopic *ptc-lacZ*. (B-B") In p[Ci]- *ci*$^{94}$ and UAS-Gap-Fu clones, strong *ptc-lacZ* was induced and (C) measured by looking at the ratio of the staining intensity of the clone compared to the AP border.

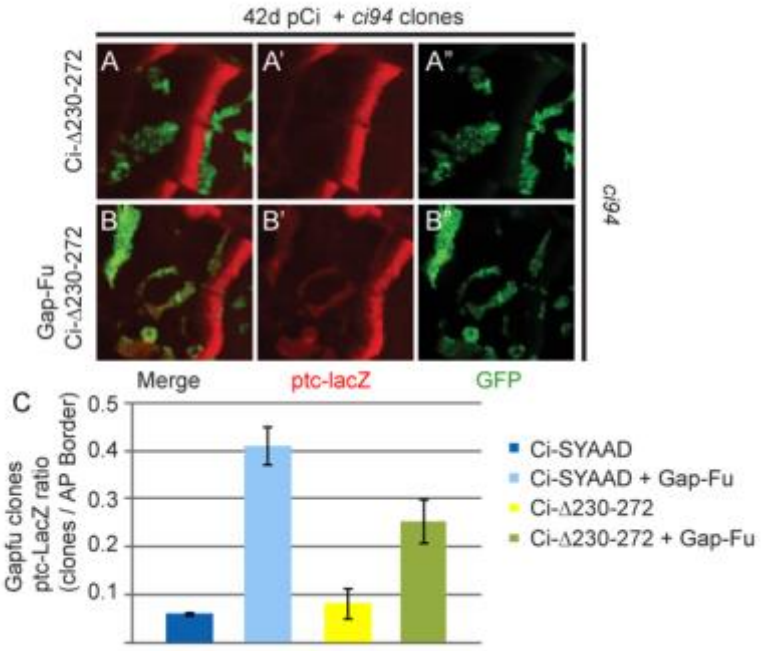


**Figure 16 gCi-Δ230-272 Has slight Ectopic Activity and Is Less Responsive to Activated Fused than gCi-SYAAD:** (A-A") In positively marked p[Ci]- *ci*$^{94}$ clones where gCi-Δ230-272 is expressed throughout the wing disc, gCi-Δ230-272 induced a small amount ectopic *ptc-lacZ*. (B-B") In p[Ci]- *ci*$^{94}$ and UAS-Gap-Fu clones, moderate *ptc-lacZ* was induced and (C) measured by looking at the ratio of the staining intensity of the clone compared to the AP border.

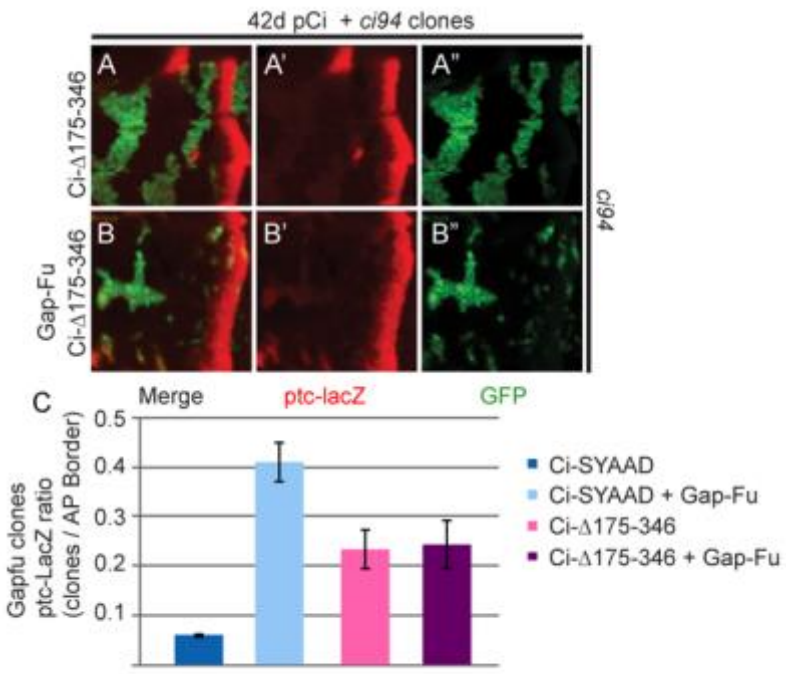


**Figure 17 gCi-Δ175-346 Has Ectopic Activity and Is Minimally Responsive to Activated Fused:** (A-A") In positively marked p[Ci]- *ci*$^{94}$ clones where gCi-Δ175-346 expressed throughout the wing disc, gCi-Δ175-346 induced moderately high ectopic *ptc-lacZ*. (B-B") In p[Ci]- *ci*$^{94}$ and UAS-Gap-Fu clones, moderate amounts of *ptc-lacZ* was induced and (C) measured by looking at the ratio of the staining intensity of the clone compared to the AP border.

***Materials and Methods***

*CRISPR Cloning*

*1st round of CRISPR:* We cloned the 5kb mini-white gene from att Pacman into the first intron of Ci in a BSK Fragment1 (from Chapter 3) using the restriction enzyme AclI. The PAM sites (green) associated with guide RNA 1 (TGG->TGA) and guide RNA 2(TGG->TTG) (blue) were mutated on this Donor Template (guide RNA 1 TCACCCAAAAATCTCGTATT TGG) (guide RNA 2 ATATATATACAAGAGTTCCT TGG) in the Ci first intron. The guide RNA's were under 100 base pairs from each end of the mini white gene flanked by 2Kb of Ci outside of each guide RNA. The guide RNA's were cloned in pU6 chiRNA vectors separately. The donor template, guide RNA 1, and guide RNA 2 were then co-injected into fly embryos from the Mann Lab that were wlig4; attp40 (nos-Cas)/cyo. We crossed the injected flies to ywflp; sp/cyo; T6/T2; y+/y+ (pearly balancer phenotype) and selected for flies that were white +.

*2nd round of CRISPR:* We balanced the Ci-white+ flies with the nanos-Cas9 expression flies creating flies that were wlig4; attp40 (nos-Cas9)/cyo; Ci-white+/y+. We developed a Donor construct with a guide RNA 3 in the 3'UTR and guide RNA 4 in the first intron, we cloned a larger BSK F1 by adding 500 bps extra on the 3'UTR region to create a 1.1 Kb homology region outside of guide RNA 3 and 2kb homology region outside of guide RNA 4 (guide RNA 3(GGG->CCG): GGGCTTACGCCGGTATTAG GGG) (guide RNA 4 (CGG-> CAG) (GCTTTGGGTGTAGGAGCGTC CGG). guide RNA 3 and guide RNA 4 were cloned into a dual U6 (1+3 promoter) expression construct pCFD4 using Gibson assembly. The donor construct and the pCFD4 plus guide RNA's were then injected into wlig4; attp40 (nos-Cas9)/cyo

Ci-white+/y+ flies. Injected flies were injected into pearly balancer where non pearly white – flies were selected. Once the white – flies were selected that should have the mutation of interest, the flies were balanced then genotyped and then PCR'ed to confirm introduced mutation.

Donor Template Cloning: Overlapping primers were used to add, mutate, and delete regions on Ci, which were then re-cloned into BSK-F1 Donor Construct using Gibson Assembly

*Immunostaining and Genomic Transgene Cloning:* See protocols from Chapter 3

Fly crosses

Females of the genotype ywflp; plz/T6; Ci94/y+ were crossed to males ywflp; sp/cyo; gCi-Wt/ΔCORD/SYAAD/Δ230-272/Δ175-346/S849A; ci94/y+ to obtain whole discs for third chromosome transgenes

Females of the genotype ywflp; plz/T6; Ci94/y+ were crossed to males ywflp; sp/cyo; crCi-Wt/ΔCORD/SYAAD/Δ230-272/Δ175-346/S849A/y+ to obtain whole discs for CRISPR transgenes

Females of the genotype ywflpUAS-GFP; tubgal8040A/cyo; C765plz/Tm6B; Ci94/y+ were crossed to males of the genotype ywflp; pka40A/cyo; gCi-Wt/ΔCORD/S849A /Tm6B to create *pka* clones in wing discs

Females of the genotype ywflpUAS-GFP; 42dp (Ci)tubgal80/cyo; C765plz/Tm6B; Ci94/y+ were crossed to males of the genotype ywflp; 42dcos/cyo; gCi-Wt /S849A /Tm6B to create *cos and ci94* clones in wing discs

Females of the genotype ywflpUAS-GFP; 42dtubgal80/cyo; C765plz/Tm6B; Ci94/y+ were crossed to males of the genotype ywflp; smo42dGapfu/cyo; gCi-Wt /S849A /Tm6B to create *UAS-Gap-Fu* clones in wing discs.

Females of the genotype ywflpUAS-GFP; 42dp (Ci)tubgal80/cyo; C765plz/Tm6B; Ci94/y+ were crossed to males of the genotype ywflp; smo42dGapfu/cyo /cyo; gCi-Wt /SYAAD/Δ230-272/Δ175-346/Tm6B to create UAS-Gap-Fu *and ci94* clones in wing discs

Females of the genotype fumH63; 42d y+ p(Fu)/cyo; *Su(fu)*C765plz/Tm6B; Ci94/y+ were crossed to males ywflp; sp/cyo; gCi-Wt /SYAAD ci94/y+ to obtain whole discs for third chromosome transgenes.

Females of the genotype fumH63; 42d y+ p(Fu)/cyo; *Su(fu)*C765plz/Tm6B; Ci94/y+ were crossed to males ywflp; sp/cyo; *Su(fu)*/Tm6B; gCi-Wt /SYAAD /y+ to obtain whole discs for CRISPR transgenes

## Discussion

We successfully created Ci "transgenes" expressed at physiological levels to investigate how Hh regulates Ci activity and stability. We found that when processing is eliminated 1) Ci-155 levels are stabilized but still undergo complete proteolysis at high levels of Hh signaling and 2) Ci activity is graded at the AP Border but has some ectopic expression independent of Hh. Activated Fused in Ci-S849A induced very high Ci activity and destabilized Ci-155 levels, similar to Ci regulation at the AP Border. It was thought that PKA and Cos2 both contributed to silencing of Ci-155 independent of processing, but we found that only Cos2 has a role in silencing Ci-155 by binding to the CORD region as well as an additional role in stabilizing Ci-155 in the cytoplasm. We confirmed *in vitro* studies that suggest Su(fu) inhibits Ci activity by binding to the Ci-SYGHI, but we found that this is not the entire binding region. Finally, we were able to offer some evidence that the key target of Fu is Ci and its target site is on Ci near the region where Su(fu) binds.

### *Ci Activity and Stability Independent of Processing*

The Hh signal promotes the elevation of Ci-155 levels by blocking processing but at the highest levels of Ci activity, Ci-155 is completely degraded which is in enhanced in Ci-S849A. It is likely that Hh regulates the graded proteolysis of Ci-155 through two mechanisms 1) induction of Rdx and 2) Su(fu)/Ci disassociation, yet direct evidence for this is limited. Rdx binds Ci at 6 cluster regions, but mutation of cluster sites "S3, S4, and S5" at specific amino acids has been shown to block binding of Ci to Rdx. Su(fu)/Ci disassociation may also contribute to graded proteolysis through the action of Fu, as was seen when we looked at activated Fu in Ci-S849A. Active Fu, expressed artificially or at the AP border, activates Ci-155 which causes Ci-155 to be

released from Su(fu), loss of Su(fu) binding to Ci-155 causing it to become less stable. To investigate how each of these processes contribute to Ci proteolysis, we have created crCi-S3-5, and crCi-S849A S3-5 transgenes that should block Rdx binding, and we will analyze their effects on Ci-155 stability with and without the *Su(fu)* mutation.

*pka* and *cos2* clones induce ectopic Ci activity in the wing disc. Since they are both involved in Ci processing, it was thought that this ectopic expression was due the elevated Ci-155 levels and loss of Ci-75 repressor. However, when you compare *pka* and *cos2* clones to *slimb* clones, Ci activity is much higher for *pka* and *cos2*. This suggests that both Cos2 and PKA have an additional role in silencing Ci-155 independent of processing.

We expected loss of *pka* to have ectopic activity in Ci-S849A but we found that *pka* clones had the same phenotype as Ci-S849A alone which may be due to muted activity of S849A. To further investigate PKA activity, we have created crCi PKA S(1-3)A, a Ci mutant that blocks PKA phosphorylation on 3 residues necessary for PKA dependent proteolysis. We will compare the activity and Ci stability between crCi PKA S(1-3)A and crCi S849A, to confirm that these PKA mutations block processing, then we will induce *pka* clones in crCi PKA S(1-3)A to determine if there is any additional activity. In Gli2 (mammalian Ci), the first four PKA target sites are responsible for processing, sites 5 and 6 are necessary for silencing activity so we are hoping to determine if the mechanism is conserved (Niewiadomski, Kong et al. 2014).

In contrast to PKA, we found that Cos2 did have a role in silencing Ci-155 independent of processing, explaining why *cos2* clones have higher activity than *slimb* clones. We hypothesized that the mechanism of Ci-155 silencing was through the binding of Cos2 to Ci-155 through the CORD domain. To test this, we blocked processing using *pka* clones and compared Ci-Wt to Ci-ΔCORD; we found that Ci-ΔCORD had higher activity than Wt, supporting our

hypothesis. One concern from this experimental design is that using *pka* clones to block processing might create some additional artifact, so we will confirm these results by look at Ci activity in crCi-S849A vs crCi-S849AΔCORD.

Su(fu) *Inhibition of Ci-155*

Even though Su(fu) is dispensable in flies, it is able to restrict Ci-155 activity. This role is only revealed when there is an additional loss of Fu kinase activity; loss of *Su(fu)* rescues the loss of *fu.* The mechanism of Su(fu) inhibition of Ci activity is not well understood but important to investigate since mammalian Su(fu) inhibition of Gli is well conserved and Su(fu) is indispensable for mammalian Hh signaling. Su(fu) binds to Ci/Gli at a conserved N-terminus SYGHI motif to restrict Ci-155 in the cytoplasm; mutating SYGHI to SYAAD blocks this binding but this has never been tested *in vivo* at physiological levels.

We found that gCi/crCi-SYAAD moderately blocked Su(fu) binding because it was able to destabilize Ci-155 and partially rescue the $fu^{mH63}$ mutation. There has been some additional evidence of a smaller C-terminus Su(fu) binding region (1370-1397) responsible for Su(fu)/Ci binding in the nucleus, blocking Ci-155 activity by preventing the binding of the CBP co-activator. We will test to see if crCi-SYAAD Δ1370-1397 can fully rescue a *fumH63* mutant, but it is more likely that Su(fu) requires a larger binding region in the N-terminus Δ230-272 or Δ175-346; these transgenes are currently being investigated.

*Ci Activation by Fused Kinase*

The mechanism for Ci activation is not well understood. We know that 1) Fu is necessary for activity, 2) Fu binds and phosphorylates both Su(fu) and Cos2, 3) Su(fu) and Cos2 restrict Ci

activity, but 4) the critical targets sites of Fu are not known. It was thought when Fu phosphorylates Cos2 or Su(fu), Ci is released from their cytoplasmic binding and transformed into a Ci-155 activator form. However, when we mutated these known critical residues on Cos2 and Su(fu), we found that they were dispensable. This suggests that the direct critical target of Fu could be another protein, which we attempted to investigate by the genetic screen in Chapter 3, or the target could be Ci-155. We were previously unable to investigate Ci as a Fu target because we couldn't study Ci variants at physiological levels.

Now that we have CRISPR and genomic Ci, we can delete/mutate the regions on Ci that are most likely involved with activation. We looked at the N terminus Su(fu) binding region because it is possible that a critical Fu target is near the SYGHI Su(fu) binding motif. We created deletions that include this motif (Δ230-272 and Δ175-346) to determine if they contained a critical Fu target site. We found that Ci-SYAAD and CiΔ230-272 but not CiΔ175-346 were activated by Fu, indicating a possible target site in this region. To investigate further, we created CiΔ175-230, CiΔ272-346, and Ci variants with serine point mutations within CiΔ230-272. These Ci constructs will not contain the Su(fu) binding motif so if they contained the critical Fu target site we would expect them do have reduced Ci activity in the whole disc and be unresponsive to activated Fu.

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# Chapter 5

# Discussion

*The Importance of Studying Genes Expressed at Physiological Levels*

Developing physiological genomic transgenes Cos2 and CRISPR variants for Ci was a difficult cloning process, but it was necessary to overcome the artifacts introduced by the UAS/Gal4 system. Using physiologically expressed genomic Costal2 (gCos2), we found that Fu binds Cos2 to support efficient Ci processing and we contradicted the evidence from multiple labs indicating that Fu phosphorylation on Cos2 sites S572 and S931 was necessary for Ci activation. Thus, we will continue to investigate the mechanism of Ci activation by looking at other possible Fu targets such as Ci-155.

UAS-Ci had many limitations explored in Chapter 4, that restricted our ability to study different Ci variants. Using genomic Ci transgenes (gCi), we learned that *ci* introns are important for producing normal Ci-155 levels and that *ci* isoform A is necessary and sufficient to reproduce normal Ci functions. Using CRISPR *ci* alleles, we are investigating how Hh signaling regulates Ci activity and stability in the absence of processing, how Su(fu) inhibits Ci-155 activity, and if Ci-155 is a direct target of Fu.

*Post-Transcriptional Regulation of Ci*

Ci and its homologues Gli2 and Gli3 are processed to an N-terminus repressor form in the absence of Hh signaling while Gli1 cannot be processed (Sasaki, Nishizaki et al. 1999) (Wang, Fallon et al. 2000). When the Hh signal is present, Gli2/3 are in their full-length activator form in addition to the expression of Gli1, which is exclusively expressed when the pathway is active (Wilson and Chuang 2010). The Gli proteins are dynamically regulated by processing, activation, and degradation similar to Ci, but also

undergo alternative splicing. Human Gli1 has two splice variants, 1) Gli1ΔN (Δ1-128aa) which cannot bind SUFU and has weak transcriptional activity and 2) tGli (Δ34-74) which is a truncated Gli1 that acquire a gain of function activity promoting tumorigenesis in cancer cells (Zhu and Lo 2010). Human Gli2 was also found to have two isoforms (Gli2ΔN and Gli2ΔC) that promoted the activator form of Gli2 and were associated with cancerous cells that promote metastasis (Sadam, Liivas et al. 2016). The post-transcriptional regulation of Gli1/2 isoforms were functionally relevant for efficient Hh signaling however comparable studies had not been investigated for Ci.

We initially began looking at post-transcriptional regulation of *ci* because of "hits" we identified from a Hh sensitized genetic screen. In the screen, we used flies that were sensitized with a loss of Fu kinase activity and a heterozygous loss of Su(fu) ( $fu^{mH63}$; $su(fu)^{LP}/+$). This genotype causes a partial fusion of veins 3 and 4 in the adult fly. The gene responsible for the intervein space between L3 and L4 is the Hh target gene collier/knot (Crozatier, Glise et al. 2003). We hypothesized we could identify novel Hh related genes that altered the partial fusion phenotype using a fly deletion library; a collection of flies with different heterozygous deletions across the second and third chromosome.

The cross would produce flies with $fu^{mH63}$, deficient of kinase activity, a heterozygous loss of Su(fu) ($su(fu)^{LP}/+$), and an additional deletion ($fu^{mH63}$/Y; deletion/cyo; 82B $su(fu)^{LP}$C765plz/TM6B). These deletions/deficiencies incorporated multiple genes and showed no visible adult wing phenotype between veins 3 and 4 but when they were crossed to a mutant $fu^{mH63}$; $su(fu)^{LP}/+$ background, many deletions further fused veins 3 and 4 or rescued the partial fusion. I investigated these deletions by

acquiring more deletions within these "hits" and determining which of these smaller deletions contained the gene responsible for altering the vein pattern as discussed in more detail in Chapter 3.

From the screen, we identified two genes, *mago nashi* from deficiency (9063) and *srp54* from deficiency (8042) which reduced pathway activity in a $fum^{H63}$; 82B $su(fu)^{LP}$C765plz /+ background and are involved with post-transcriptional regulation. We focused particular attention on *ci* as a target of these "splicing" regulators. *ci* is on the fourth chromosome in a heterochromatin region; it consists of six exons with a large first intron and three theoretical isoforms: isoform A which is routinely studied in Hh signaling, isoform B which lacks the first 119 amino acids, and isoform C which only contains exons 2-5 (Flybase). Isoform A is the only variant that has been studied, it has never been shown if isoform B and C exist, if they are functionally relevant, nor is it known if the post-transcriptional regulation of *ci* can affect Hh signaling activity. Using the Drosophila wing disc, we investigated the potential for *ci* to be post-transcriptionally regulated.

We found that both Mago and Srp54 knockdown affected *ci* RNA and protein levels; Mago RNAi induced an altered the *ci* splice pattern and lowered Ci-155 protein levels while Srp54 RNAi reduced total *ci* RNA levels and had higher expression of Ci-155 in the anterior. The protein elevation in Srp54 knockdown is most likely due to impaired Ci-processing and is independent of Srp54 RNAi reduction of *ci* RNA levels. It seems that Srp54 contributes to efficient post-transcriptional regulation of *ci* in addition to other roles affecting the output of Hh signaling.

The EJC in Drosophila consists of Mago Nashi (MGN), Tsunagi (Tsu), Eif4AIII, and Barentsz (Btz) (Degot, Le Hir et al. 2004) (Palacios, Gatfield et al. 2004) (Macchi, Kroening et al. 2003), which support mRNA regulation involving nuclear export (Le Hir, Gatfield et al. 2001). Since Mago is member of the EJC, we looked to see if the complex as a whole was involved with *ci* post-transcriptional regulation. We knocked down each member of the EJC to determine if they had a comparable effect to Mago. We found that eIF4AIII, and Y14 which are nuclear components of the EJC and involved with splicing, had reduced Ci-155 levels and lower Ci activity in a *fu* sensitized background they were knocked down. Btz, which is cytoplasmic, did not show any reduction in Ci-155 levels or Ci activity when it was knocked down, thus confirming a role for the nuclear EJC components in regulating *ci* RNA.

To confirm that Mago and Srp54 were acting on *ci* RNA cell autonomously, we created MARCM clones that expressed either Mago or Srp54 RNAi. Clones of the RNAi alone were difficult to make because they were lethal to the individual cells, but were surprisingly healthier when they were coupled to an altered Hh signaling component that produces ectopic activity. Mago and Srp54 RNAi reduced ectopic activity induced from mutant *pka* clones and clones with activated Fu (Gap-Fu), which indicates that the RNAi is working cell autonomously to affect *ci* RNA levels which results in reduced Hh pathway activity.

We further investigated *ci* RNA regulation by identifying whether the protein products of isoforms A and B exist in the fly wing disc. We found that isoform B exists but in very small amounts compared to the highly-expressed isoform A. We looked at the functions of each isoform by mutating the ATG start codon for isoform A (gCi ATG-A)

which produces Ci-B and mutating the ATG start codon for isoform B (gCi ATG-B) which produces Ci-A. Ci-A was similar to wild type while Ci-B only showed a deficit in repressor function due the lack of N-terminus. It does not appear that Ci-B has any relevant role in Hh signaling because it is weakly expressed and does not have an obvious role affecting Ci activity or Ci-155 levels.

Despite not finding an obvious functional role for Ci-B, it is possible that efficient splicing of Ci-A is important for Hh signaling. To determine if there is regulated splicing, we looked at gCi transgenes that could not be spliced such as gCi-SV1, intronless Ci with a generic SV40 3'UTR. We found that gCi-SV1 had normal Ci activity but lower Ci-155 levels similar to the phenotype we saw with Mago knockdown. Furthermore, Mago knockdown did not further reduce Ci-155 levels of gCi-SV1 compared to gCi-Wt, indicating that they are reducing Ci-155 levels through the same mechanism. We suggest then that efficient *ci* RNA splicing is necessary for normal Ci-155 protein levels.

Interestingly, lower Ci-155 proteins (up to 2-fold) levels do not affect Ci activity when there is optimal Hh signaling and will not reduce Ci activity at the AP border of wild-type discs. However, when there is sub-optimal activity such as in a $fu^{mH63}$; $su(fu)^{LP}$/+ mutant or in ectopic expression from activated Fu or loss of *pka*, we found that lower Ci-155 levels result in lower Ci activity. On the other hand, we found that excessively high levels of Ci-155, expressed by a UAS-Ci cDNA has a dominant negative effect on Ci activity and reduces the level of Hh target genes. These findings confirm that physiological expression and maintaining normal levels of Ci-155 is necessary for Hh signaling and studying Ci variants.

### *Ci Activity and Stability in the Absence of Processing*

We used the Ci variant Ci-S849A, which blocks Slimb binding and subsequent processing to the Ci-75 repressor form, expressed at physiological levels to understand how Ci levels and activity are effected in the absence of processing. We found that physiologically expressed Ci-S849A caused the stabilization of Ci-155 throughout the wing disc independent of Hh signaling. It also appeared that processing was not necessary for maintaining graded Ci activity at the AP border. There was high ectopic *dpp* expression and some ectopic *ptc-lacZ* expression throughout the anterior. This supports the evidence that Ci-75 repressor negatively regulates *dpp* expression and potentially *ptc* expression, but ectopic ptc-lacZ may also be the result of unusually high Ci-155 levels(Muller and Basler 2000) .

There was graded proteolysis of Ci-155 at the AP border in whole Ci-S849A wing discs. This suggests that the decline in Ci-155 levels at the AP border is not due to Hh regulated processing, but due to Hh regulated proteolysis. This degradation is seen at the highest levels of signaling as further confirmed by looking at UAS-Gap-Fu clones in Ci-S849A wing discs which had extremely high *ptc-lacZ* expression and decreased Ci-155 levels. There are two potential mechanisms that result in the decrease of Ci-155 levels: Su(fu)-inhibited proteolysis and Hib-dependent proteolysis. At the AP border, Hh induces the expression of Hib which binds Ci-155 promoting its proteolysis through Cul3 ubiquitin ligases. Su(fu) is expressed throughout the wing disc but is down regulated at the AP border by Hib; loss of Su(fu) decreases Ci-155 levels throughout the anterior and the AP border. Since Hib is not expressed in the anterior, the mechanism Ci-155 degradation by loss of Su(fu) is also independent of Hib.

Therefore, at the AP border, it is not clear to what extent each mechanism contributes to Ci-proteolysis. One possibility is at high levels of Hh, Fu is very active which limits the interaction between Su(fu) and Ci-155, this in turn leads to an unbound and unstable Ci-155 that gets targeted for proteolysis. Hib has also been shown to indirectly down-regulate Su(fu) protein further promoting Ci instability. In order to dissect the mechanism of Su(fu)-mediated proteolysis vs Hib-mediated proteolysis we created Ci variants with altered Su(fu) and Roadkill binding as discussed in Chapter 4.

*Cos2 on Ci-155 Activity*

We compared *ci vs ci/cos2* clones in the Ci-S849A wing discs which showed elevated *ptc-lacZ* and decreased Ci-155 levels in *ci*/*cos2* clones compared to *ci* clones. This indicates that there is an additional role for Cos2 to silence Ci-155 and stabilize Ci-155 levels. Cos2 binds to three regions on Ci-155 (Zn, CDN, and CORD), we hypothesized that Cos2 silences Ci-155 by binding to one of these regions(Zhou and Kalderon 2010).We investigated whether CORD was the critical region by creating *pka* clones (to block processing) in Ci-Wt and Ci-ΔCORD and found that there was a higher expression of *ptc-lacZ* in Ci-ΔCORD supporting the hypothesis that Cos2 binds to Ci-155 CORD regions in silence Ci-activity but this will continue to be investigated. We will induce *cos2* clones in Ci-S849A and Ci-S849AΔCORD flies. If Cos2 silences Ci-155 in the absence of processing, we would expect *cos2* clones to have elevated ptc-lacZ in the clone compared to the rest of the wing disc in Ci-S849A. We would expect comparable levels in the clone to the rest for the anterior for Ci-S849AΔCORD because we would expect to see higher ectopic *ptc-lacZ* throughout the entire anterior of the wing disc.

gCos S182N blocks nucleotide binding as well as binding to Ci-155 through the CORD region. gCosS182N showed elevated *ptc-lacZ* levels, further supporting that Cos2 binding to the CORD region silences Ci activity. We also found that gCosS182N had elevated Ci-155 levels indicating that there was impaired processing. It was previously shown with UAS transgenes that CosS182N had impaired processing but could be rescued by the additional loss of the CORD domain (Zhou and Kalderon 2010). This suggests that Cos2 binding to the CORD domain also supports efficient processing. However, since we saw that CiΔCORD is relatively normal and comparable to wild-type discs, this function is probably redundant with the other Cos2 binding region (CDN and Zn) and that the processing defects seen with CosS182N may be due to its role in nucleotide binding.

*Costal and Fu Binding*

We found that Cos2 recruits Fu to the processing complex and that this interaction is necessary for efficient processing (Zadorozny, Little et al. 2015). Fu alleles with a truncated C-terminus are unable to bind Cos2; they have elevated Ci-155 levels but are able to form some repressor based on the *hh-lacZ* Ci-75 reporter assay. Physiologically expressed Cos2 with a deleted Fu binding region (gCos-ΔFu) also had elevated Ci-155 levels but was still able to form Ci-75 repressor. The data from the truncated *fu* alleles and gCos-ΔFu indicate that Cos/Fu binding supports a small amount of processing indicated by the *hh-lacZ* reporter assay but not efficient processing as indicated by elevated Ci-155 levels.

gCosΔFu was unable to support high levels of Ci activation at the AP border in both whole wing discs and clones. This suggests that when Cos2 cannot bind Fu, Fu

molecules fail to aggregate which prevents their ability to cross-phosphorylate the Fu activation loop, necessary for full Fu activation. In the presence of Hh signaling, activated Smoothened (Smo), promotes the translocation of the Cos2 and Fu bound complex to the membrane for Ci activation, but failure to bind to Fu causes a reduction in Hh target gene expression. Furthermore, loss of Cos2 causes the destabilization of Fu, which supports the assumption when Fu cannot bind to Cos2, its full activation is compromised which is evident by *cos2* and gCosΔFu clones at the AP Border.

*How does Su(fu inhibit Ci-155?*

It was thought that Su(fu) restrains Ci-155 in the cytoplasm and restricts its access to the nucleus because loss of Su(fu) results in Ci-155 nuclear localization (Methot and Basler 2000). In this paper, the UAS-Ci transgene had mutated PKA sites (UAS-$Ci^{PKA4}$) in order to study the full-length form of Ci-155. UAS-$Ci^{PKA4}$ was expressed with a C765 Gal4 driver and induced ectopic *ptc-lacZ* throughout the anterior (Fig 1A). This was rescued by the co over-expression of UAS-Su(fu), which indicates that Su(fu) can inhibit full-length Ci activity (Figure 1B). To investigate whether Su(fu) inhibits Ci in the cytoplasm vs the nucleus, $Ci^{PKA4}$ was ubiquitously expressed throughout the fly. Mutant $su(fu)^{LP}$ and wild type $Ci^{PKA4}$ flies were treated with Leptomycin B(LMB), which blocks nuclear export, to determine the localization Ci-155 and *ptc-lacZ* expression in the salivary gland. In wild type $Ci^{PKA4}$ flies, Ci-155 was expressed throughout the nucleus and cytoplasm; *ptc-lacZ* was slightly elevated in the nucleus. In mutant $su(fu)^{LP}$ $Ci^{PKA4}$ flies, Ci-155 and *ptc-lacZ* were highly expressed in the nucleus, indicating that Su(fu) inhibits Ci activity by restricting its access to the nucleus.

We believe this data is weak and suspicious for two main reasons: 1) if it was possible to ubiquitously express $Ci^{PKA4}$, why couldn't this experiment be performed using the AP border in wing discs where Hh signaling is present and perform LMB treatment with and without Su(fu) and 2) in the salivary gland, if normal Hh signaling is normally regulated, I would have expected there to be nuclear accumulation of Ci-155 in the wild type image because when processing is blocked such as in $Ci^{PKA4}$ or *cos2* clones, Ci 155 is more nuclear.

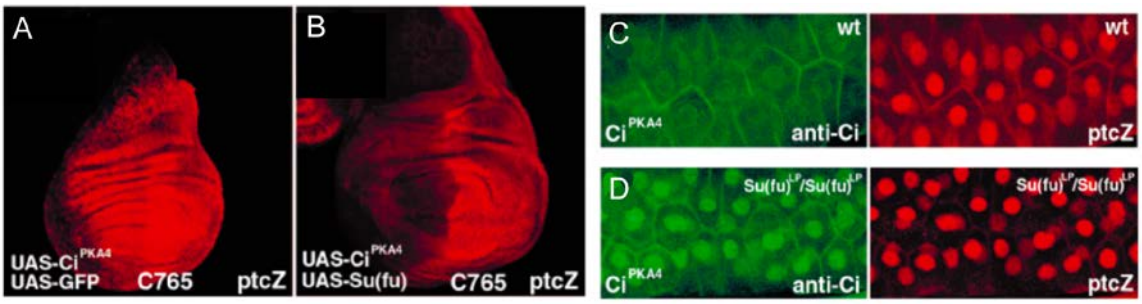


**Figure 1 Su(fu) Inhibits Ci activity:** (adapted from Methot and Basler, 2000) (A) Expression of UAS-$Ci^{PKA4}$ and UAS-GFP with C765 Gal4 driver shows that there is ectopic *ptc-lacZ* throughout the anterior of the wing disc with UAS- $Ci^{PKA4}$. (B) Expression of UAS-$Ci^{PKA4}$ with UAS-Su(fu) reduces ectopic *ptc-lacZ* throughout the anterior of the wing disc because Su(fu) inhibits active Ci-155. (C) Ubiquitously expressed $Ci^{PKA4}$ expresses Ci-155 evenly in the cytoplasm and the nucleus with moderate activation of *ptc-lacZ* in the nucleus

Further evidence suggested Su(fu) was able to inhibit Ci-155 through cytoplasmic anchoring by binding to the N-terminus of Ci-155 (Wang, Amanai et al. 2000). In this study, UAS-Ci was expressed with an MS1096 driver in the wing disc to look at

cytoplasmic vs nuclear localization of Ci-155 following LMB treatment. The study claimed that in wild type discs, Ci-155 was cytoplasmic, but when they removed Su(fu) or the N-terminal Su(fu) binding region on Ci, Ci-155 was nuclear, supporting the evidence that Su(fu) inhibits Ci activity through cytoplasmic anchoring.

However, the images aren't very convincing, the cells in a wing disc are very small and the study used a low magnification to image the wing discs. It is also unclear how they came to this conclusion because Ci was expressed under non-physiological levels. Recently this lab reported an additional Su(fu) binding region in the C-terminus of Ci-155 (1370-1397) that is apparently necessary for Su(fu) binding in the nucleus to block the Ci-155 coactivator from binding but this evidence is also based exclusively on inconclusive overexpression of UAS-Ci transgenes and cell culture experiments (Han et al., 2015).

In contrast, the Beachy Lab used Drosophila cl8 cells to study the effects of Cos2 and Su(fu) on cytoplasmic retention of Ci-155. They found that co-transfection of Ci with Cos2 did restrain Ci-155 to the cytoplasm but not with co-transfection of Su(fu) unless it was at very high levels (Chen, von Kessler et al. 1999). The Holmgren lab also reported nuclear localization of Su(fu) with Ci at high levels of Hh signaling. Following LMB treatment of UAS-Myc tagged Su(fu) and UAS-Ci, Sufu and Ci were cytoplasmic. Following LMB treatment of UAS- Su(fu) and UAS-Ci, Sufu and Ci were nuclear, indicating that Myc-tagged Sufu gave artificial cytoplasmic retention (Sisson, 2006).

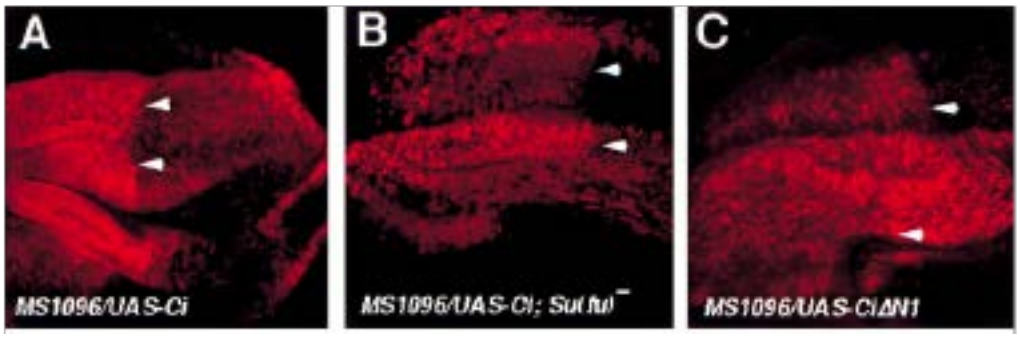


**Figure 2 LMB treatment of Wing Discs Used to Determine the Nuclear Localization of Ci-155:** (adapted from Wang et al., 2000) (A) UAS-Ci was driven by an MS1096 driver and Ci-155 levels were stained in red. It was determined that Ci-155 was cytoplasmic after LMB treatment. (B) UAS-Ci was driven by an MS1096 in mutant $su(fu)^{LP}$ flies and stained with Ci-155 in red. It was determined that Ci-155 is nuclear after LMB treatment. (C) UAS-CiΔN (loss of Su(fu) binding) was driven by an MS1096 driver and Ci-155 levels were stained in red. It was determined that Ci-155 was cytoplasmic after LMB treatment.

Since there is a mixture of weak and contradictory evidence describing the mechanism of Su(fu) inhibition of Ci-155, we are investigating how Su(fu) inhibits Ci-155 using physiologically expressed transgenes. We altered the conserved binding motif SYGHI in the N-terminus of Ci by mutating it to SYAAD which was reported to prevent binding of Su(fu) to Ci-155 (Zhang, Fu et al. 2013). We found that this region is not sufficient for Su(fu) to fully inhibit Ci activity because it did not fully rescue mutant $fu^{mH63}$. A potential weaker C-terminus binding region (1370-1397) was identified for Su(fu) binding to Ci in the nucleus to block the actions of co-activator CBP (Han, 2015).

We suspect that a larger binding region or an additional binding domain is required for Su(fu) to inhibit the activity of Ci-155 which is why we are currently testing physiological Ci transgenes Ci-Δ230-272, CiΔ175-346, and Ci-Δ1370-1397. We will identify which regions on Ci encompass the entirety of the Su(fu) binding region by determining which Ci variant can fully rescue the loss of Fu kinase activity. Then we will determine if Su(fu) inhibits Ci-155 by 1) cytoplasmic anchoring 2) recruitment of co-repressors, or 3) by blocking an activator protein. We will use a CiΔSufu binding transgene and transpose the entire Su(fu) binding region to another location. If the transposed region can rescue the CiΔSufu variant, then it suggests that Su(fu) acts a cytoplasmic anchor or recruits a co-repressor but if cannot rescue the Ci variant, then Su(fu) may inhibit Ci-155 by blocking the access to co-activator.

*How does Fu activate Ci-155*

We do not know the mechanism of Ci-155 activation. We do know that Fu relieves Ci-155 inhibition by Su(fu) which is why we originally thought that the necessary target of Fu was Su(fu). Zhou and Kalderon found Fu dependent phosphorylation sites Su(fu), S321 and S324, in Drosophila Kc cells co-transfected with Cos2 and FuEE; altering these sites prevented a Su(fu) gel mobility shift (Figure 3). To test whether these were the critical target sites of Fu, Zhou and Kalderon looked at whether UAS-Su(fu)-Wt or UAS-Su(fu)AA could compensate for the loss of Su(fu) in $fu^{mH63}$; $su(fu)^{LP}$/ + wing discs. If these were the critical targets of Fu, then there would not be a rescue of Su(fu) function in the Su(fu) mutant, but UAS-Su(fu)AA and UAS-Su(fu)-Wt rescued loss of Su(fu) function by reducing *ptc-lacZ* expression at the AP Border.

Thus Su(fu) does not appear to be the necessary target for Ci activation by Fu (Figure 3) (Zhou and Kalderon 2011).

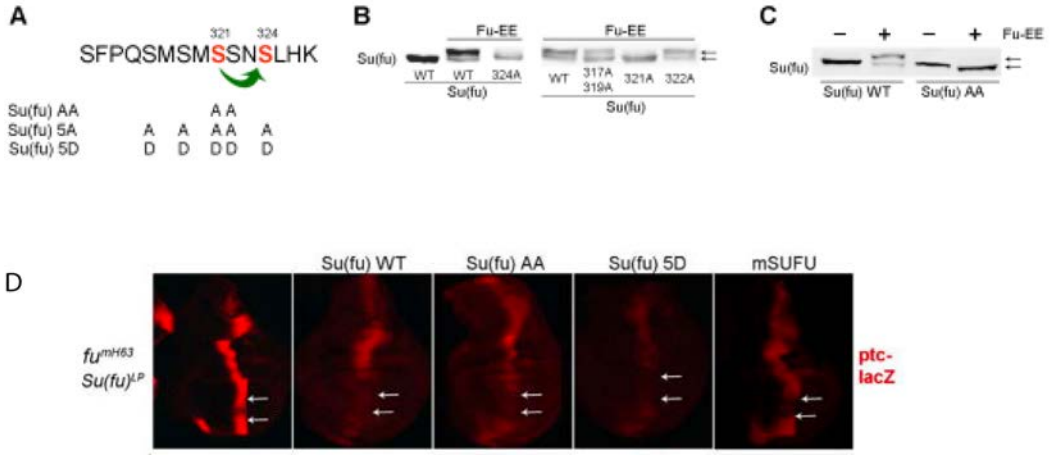


**Figure 3 Su(fu) is Phosphorylated by Fu at S321 and S324, But Not Physiologically Relevant:** (adapted from Zhou and Kalderon, 2011) (A) Diagram of Su(fu) phosphorylation motif indicating sites on Su(fu) that Fu phosphorylates. (B-C) Mutation of sites S321 and S324 to alanines blocked phosphorylation by activated Fu (Fu-EE). (D) $fu^{mH63}$; $su(fu)^{LP}$/ + wing discs have moderate elevation of ptc-lacZ at the AP Border. (D'-D") $fu^{mH63}$; $su(fu)^{LP}$/ + wing discs with UAS-Su(fu)-Wt or UAS-Su(fu)-AA driven by the C765 Gal4 driver inhibit Ci activity. Since Su(fu)-AA can inhibit Ci activity, we suspect that Su(fu) is not the critical target for Fu phosphorylation necessary for Ci activation.

Cos2 is phosphorylated by Fu at sites S572 and S931 (Nybakken, Turck et al. 2002) (Ranieri, Ruel et al. 2012). The Therond group developed phospho-antibodies against each of these phosphorylation sites and found that Hh promotes the

phosphorylation of site S931 at the highest levels of Hh signaling and site S572 at moderate to high levels of Hh signaling. A ptc-luciferase assay in cl8 Drosophila cells demonstrated that mutation of these sites to alanine reduced Hh signaling activity (Fig 4).

If Cos2 were the critical target of Fu for Ci-155 activation, we would expect that if these sites were mutated in a physiological setting signaling, the AP border would resemble a $fu^{mH63}$ disc. We used genomic Costal2 (gCos2) and mutated each site individually and together. Surprisingly we found that these discs resembled wild-type discs and rescued the flies to adulthood implying that Costal2 is not the essential substrate for Ci activation as discussed in Chapter 2.

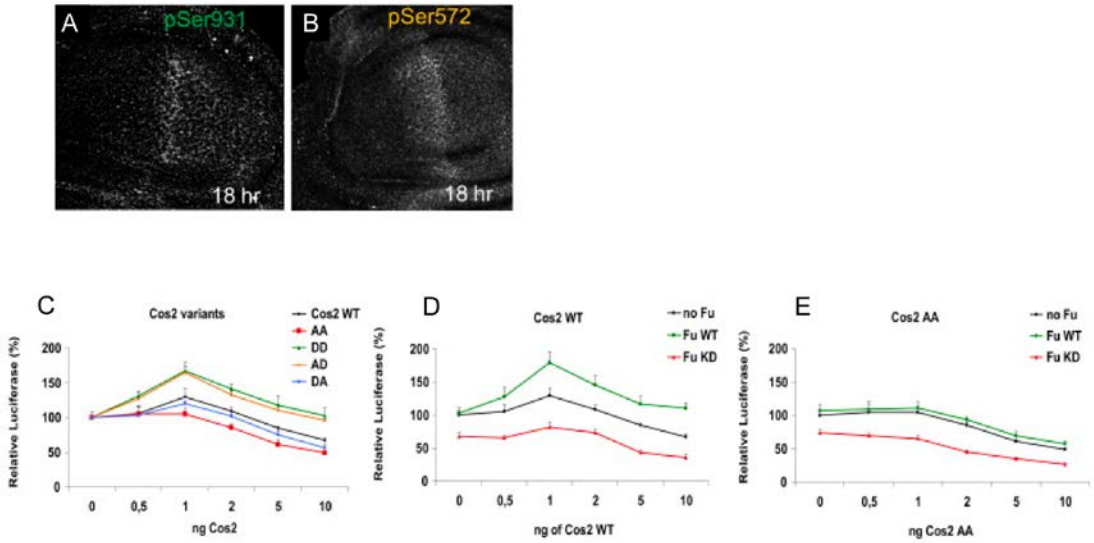


**Figure 4 Cos2 is Phosphorylated by Fu at S931 and S572:** (adapted from Ranieri and Therond, 2012) (A) Phosphorylation of Cos2 at S931, stained with a specific phospho-Cos2 S931 antibody is elevated at the AP Border. (B) Phosphorylation of Cos2 at S572, stained with a specific phospho-Cos2 S572 antibody is elevated at the AP Border and further anterior than S931, which suggests that S931 is phosphorylated is important at

high levels of Hh signaling and S572 is phosphorylated at moderate levels of Hh signaling. (C) Cos2 S572A and S931A (AA) had reduced Hh signaling measured by an *in vitro* ptc-luciferase assay while phosphomimicked Cos2 S572D and S931D had elevated expression of ptc luciferase. (D-E) Cos2 Wt had elevated ptc-luciferase expression with wild type Fu kinase activity but this was not seen for Cos AA. This data suggests that phosphorylation of these sites promotes Hh signaling activity at non-physiological levels.

Thus, we considered that Cos2 and Su(fu) served as redundant substrates for Fu phosphorylation. We analyzed what the effect of mutant gCos2 and loss of Su(fu) had on pathway activation but mutant flies had normal wing discs and grew up to be healthy adults. This leads us to conclude that there is another Fu target required for Ci activation. We hypothesized that there could be another unidentified protein which we address in the Chapter 3 with the genetic screen or the direct target of Fu could be Ci. To investigate Ci as a direct target we looked at deletions and point mutation near the Su(fu) and Cos2 bind regions as attractive targets.

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